A rise in nuclear calcium translocates annexins IV and V to the nuclear envelope

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Abstract Following incubation of human fibroblasts with Ca^{2+} ionophore A23187, we found strong immunofluorescence labelling of the nuclear envelope by annexin IV antibody. Using confocal imaging of cells loaded with Fluo-3, we showed that A23187 generates an intense and sustained rise of Ca^{2+} in the nucleus. By contrast, stimulation without extracellular Ca^{2+} produces only a brief rise in nuclear Ca^{2+} that does not promote annexin IV translocation to the nuclear envelope, and compounds that induce only a transient increase of nuclear Ca^{2+} do not support translocation of annexin IV. In addition, annexin V was also translocated to the nuclear envelope by A23187, but distribution of annexins I, II, VI and VII is unaffected. In in vitro assays with isolated nuclei, annexin V was also found to bind to the nuclear envelope in a Ca^{2+}-dependent manner. These results demonstrate that the translocation to the nuclear envelope of different types of Ca^{2+}-regulated proteins is directly triggered by a major rise of Ca^{2+} in the nucleus.

Key words: Annexin; Ca^{2+}; Nucleus; 85-kDa cytosolic phospholipase A_2

1. Introduction

The annexins (Anxs) constitute a family of Ca^{2+}-dependent phospholipid-binding proteins including 13 members with a similar structure, characterized by the presence of four or eight repeats of a 70-amino-acid segment and a variable N-terminal extremity (for recent reviews see [1–3]). A main property they share is their ability to bind to negatively charged phospholipids in the presence of Ca^{2+}, which appears to be responsible for their anti-phospholipase, anti-coagulant, anti-inflammatory, and anti-protein kinase C activities, as well as their capacity to drive Ca^{2+}-dependent aggregation of secretory granules. In addition, various annexins possess a transmembrane Ca^{2+} channel activity. The annexins have also been implicated in the regulation of endosomal vesicle trafficking and in cell-matrix interactions. Nevertheless, these many properties have complicated any interpretation as to the specific physiological role of these proteins. In addition, and despite abundant demonstrations of the Ca^{2+}-dependent binding of the annexins to phospholipid in vitro, whether this interaction also occurs within the cell is still a matter of debate, since most annexins seem to require supraphysiological Ca^{2+} concentration to bind to membrane.

In this report, we have used high-resolution confocal fluorescence microscopy to study the intracellular localization of various annexins in human fibroblasts stimulated by various Ca^{2+} agonists. We have observed that Anxs IV and V selectively translocate to the nuclear envelope during stimulation with calcium ionophore A23187, similarly to the 85-kDa cytosolic phospholipase A_2 (cPLA$_2$) [4,5]. To gain insight into the mechanism of this translocation, we have studied the intracellular calcium concentration ([Ca^{2+}]) by confocal fluorescence microscopy of cells loaded with Ca^{2+}-sensitive probes. We have found that A23187, unlike agonists that do not promote annexin translocation, induces a significant rise in nuclear [Ca^{2+}], thereby indicating that the local [Ca^{2+}] in the vicinity of the nuclear envelope regulates the translocation of Anxs IV and V, and most likely cPLA$_2$.

2. Materials and methods

2.1. Cell culture, antibodies and chemicals

Human IMR-90 fibroblasts were from ATCC (CCL-186, Rockville, MD). Monoclonal antibodies against Anxs I, II, IV and VI were from Zymed (San Francisco, CA). Rabbit anti-human Anx V antiserum was raised as described [6]. Antibodies to Anx VII were developed at the NIH. Indo-1 and Fluo-3 were from Molecular Probes (Eugene, OR), A23187 and bradykinin from Calbiochem (La Jolla, CA).

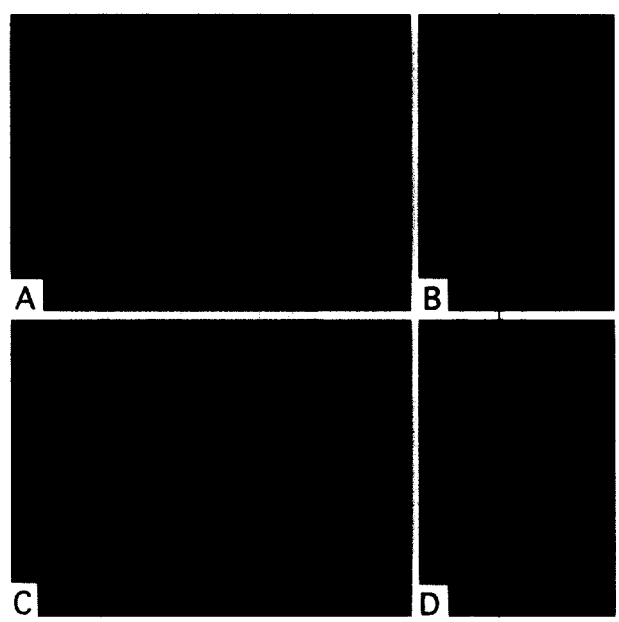

Fig. 1. Intracellular localization of Anx IV upon Ca^{2+} ionophore stimulation. Human fibroblasts were incubated in DMEM without (A,B) or with 5 μM A23187 (C,D) for 2 min at room temperature. They were then briefly washed with PBS, fixed and processed for Anx IV immunofluorescence. (B,D) Higher magnification of the cells, showing very distinctive labelling patterns in the nucleus region between control (B) and treated (D) cells.

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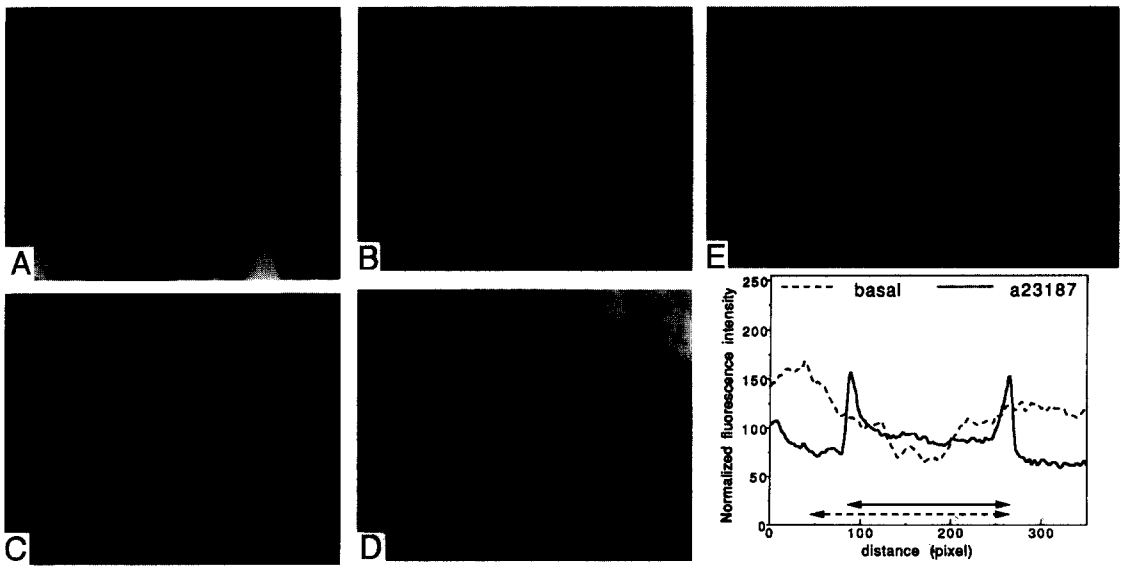

Fig. 2. Differential effect of Ca^{2+}-mobilizing agonists on Anx IV translocation and dependence on Ca^{2+} entry. Cells were incubated in DMEM for 2 min at room temperature with different agonists, then fixed and processed for Anx IV immunofluorescence. (A) No treatment; (B) 5 μM A23187; (C) 5 μM bradykinin; (D) cells treated with A23187 and then incubated for 1 min in Ca^{2+}-free medium; (E) one-dimensional intensity profile plot (bottom) of the nucleus and adjacent cytoplasm of a control (top left) and an A23187-treated (top right) cell. The black vertical bars in the images indicate the position and the width of the intensity profiles. The double-headed arrows indicate the position of the nucleus in the profiles of the control (dotted line) and the A23187-treated cell (continuous line).

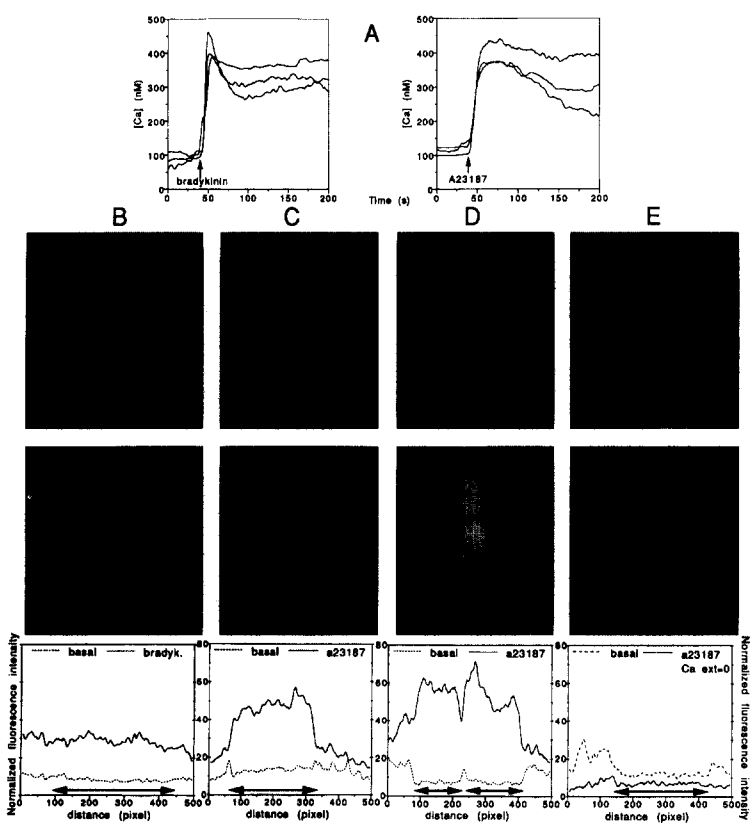

Fig. 3. Conventional and confocal microscopy analysis of intracellular free $[Ca^{2+}]$ in human fibroblasts stimulated by Ca^{2+}-mobilizing agonists. (A) Measurement of intracellular $[Ca^{2+}]$ using conventional microfluorimetry in Indo-1-loaded fibroblasts. The figure displays recordings from 3 different cells stimulated with 5 μM bradykinin (left) or 5 μM Ca^{2+} ionophore (right). (B–E) Confocal microscopy analysis of bradykinin (B) and A23187 (C–E)-induced changes in cytoplasmic and nuclear $[Ca^{2+}]$ in Fluo-3-loaded cells. Extracellular $[Ca^{2+}]$: A–D, 1.8 mM; E, <50 nM; (top) cells before stimulation; (middle) same cells after 2 min of incubation with the Ca^{2+} agonists; (bottom) plots of the fluorescence intensity profiles of the images. The profiles were determined using Biorad image analysis software (COMOS), along the y-axis in the center of the image over 1/10th of the total image width (50 pixels). The double-headed arrows indicate the position of the nuclei. Dotted line, unstimulated cells; continuous line, same cells after 2 min of stimulation.

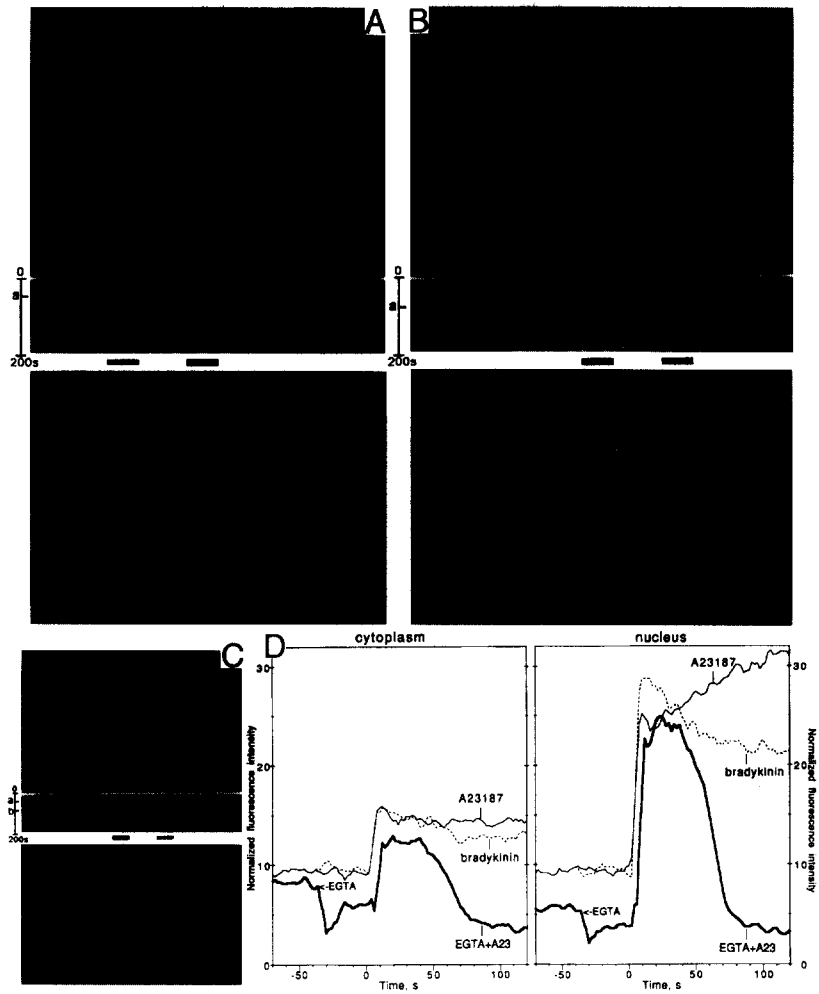

Fig. 4. Time course of agonist-induced changes in cytoplasmic and nuclear [Ca^{2+}]. Confocal images of Fluo-3-loaded cells were acquired before stimulation (A–C, top image). Then, a single line scan positioned as indicated by the white line across the cell was acquired every 2 s during 200 s. During this process, the following agonists were added: (A) a, 5 μM bradykinin; (B) a, 5 μM A23187; (C) a, EGTA 10 mM; b, 5 μM A23187. The middle image in A–C displays the fluorescence intensity of the 100 line scans generated from time 0 (top) to 200 s (bottom). The lower image shows the same cells at the end of the 200 s line scan acquisition. (D) Time course of [Ca^{2+}] in the cytoplasm and nucleus. The intensity profiles of the images generated by the 100 sequential line scans were determined for cytoplasmic and nuclear areas, indicated by black bars at the bottom of the line scan images. Left, cytoplasm; right, nucleus; dotted line, addition of 5 μM bradykinin (from A); plain line, addition of A23187 (from B); bold line, addition of EGTA 10 mM then A23187 (from C). In D only, time 0 indicates the addition of the Ca^{2+} agonist. Magnification in A is identical to that in B.

2.2. Cell Incubation and microscopy methods

For immunofluorescence studies, the cells were grown in 8-well chamber slides (Lab-Tek, Nunc). After rinsing the cells with serum-free DMEM, they were treated at room temperature with either DMEM (control) or DMEM containing a Ca^{2+} agonist. At the end of the incubation, the cells were washed for exactly 5 s with PBS, and then fixed with 3% paraformaldehyde in PBS for 15 min. The cells were then processed for Anx immunofluorescence [7]. Measurements of [Ca^{2+}] in Indo-1-loaded cells using conventional microscopy was determined as described [8]. Imaging of intracellular [Ca^{2+}] of Fluo-3-loaded cells and annexin immunofluorescence was achieved with a confocal fluorescence scanning system (MRC600, BioRad, Cambridge, MA) equipped with an argon/krypton laser and a fluorescein filter cartridge (488 nm), and attached to an upright microscope (Optiphot, Nikon). An oil immersion 60× objective was used for image acquisition of immunofluorescent samples, with a vertical resolution of 0.6 μm (reflectance mode). The immunofluorescence images displayed were obtained by accumulation of 30 scans in the photon counting mode. Images of Fluo-3-loaded cells were acquired with a water immersion 40× objective (Nikon), with a vertical resolution of 1.5 μm. The images were obtained by accumulation of 20 scans in the photon counting mode over a 20 s period. The line scan interval was 2 s.

2.3. In vitro binding of Anx V to isolated nuclei

Isolation of rat liver nuclei using sedimentation through a sucrose cushion was achieved as described by Nicotera et al. [9]. Anx V was isolated from human placenta using a standard procedure. The binding of Anx V to isolated nuclei at various [Ca^{2+}] was achieved using a Ca^{2+}-buffering solution: 50 mM Tris pH 7.5, 25 mM KCl, 5 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM EGTA. Solutions containing different free [Ca^{2+}] were obtained by adding limited amounts of a Ca^{2+} stock solution and free [Ca^{2+}] was determined using a Ca^{2+} electrode (Orion, Boston, MA). For immunofluorescence analysis, isolated nuclei were attached on poly(L-lysine)-coated glass slides and incubated for 5 min at room temperature with 500 ng of purified Anx V in various Ca^{2+}-buffered solutions. Then, nuclei were washed, fixed and processed for Anx V immunofluorescence. For immunoblotting analysis, 100 ng of purified Anx V were incubated with an amount of nuclei equivalent to 40 μg of total nuclear proteins in a solution of deter-

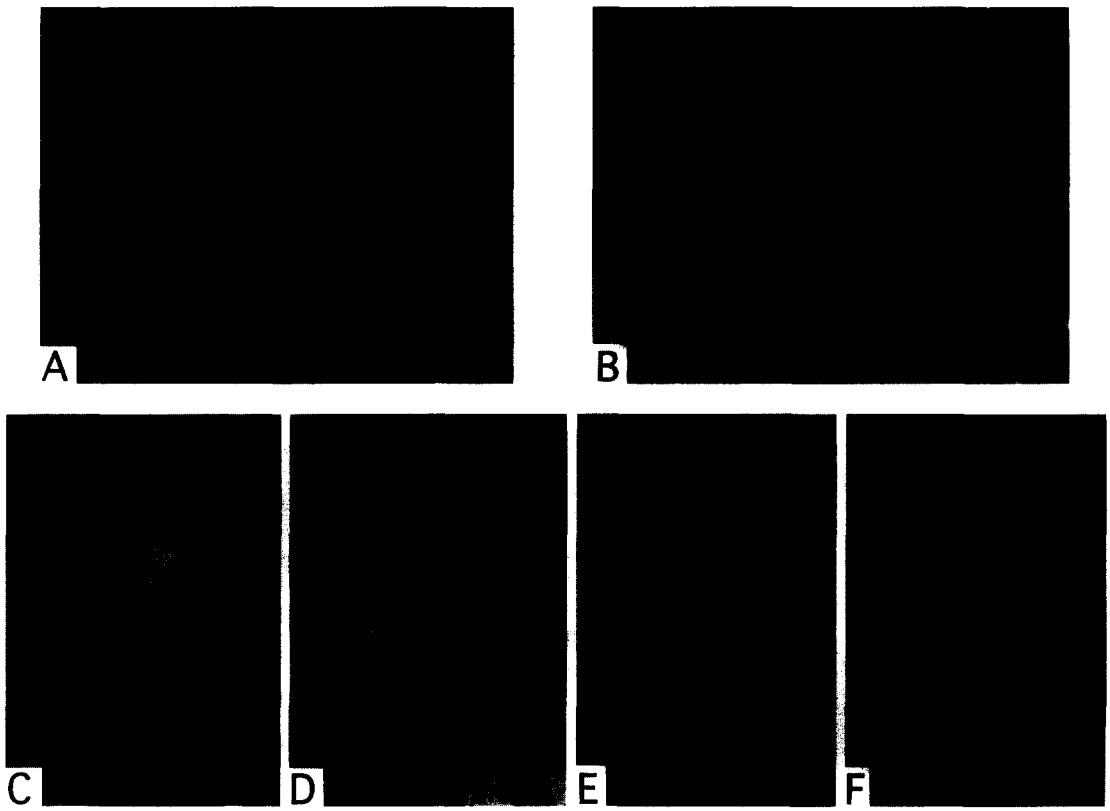

Fig. 5. Differential localization of the Anxs during A23187-induced Ca^{2+} mobilization. Human fibroblasts were stimulated for 2 min with 5 μM Ca^{2+} ionophore, then fixed and processed for immunofluorescence for Anx V (A,B), Anx I (C,D) and Anx VI (E,F). (A,C,E) Control; (B,D,F) stimulated.

mined [Ca^{2+}]. After centrifugation at 13000×*g* for 5 min, the supernatants and pellets were immunoblotted with anti-Anx V antibody and goat ^{125}I-anti-rabbit IgG (ICN, Costa Mesa, CA) and analyzed with a PhosphorImager (Molecular Dynamics).

3. Results

3.1. Intracellular localization of Anx IV upon Ca^{2+} mobilization

Immunofluorescence staining of Anx IV in unstimulated human fibroblasts IMR-90 shows that this annexin is localized in a diffuse cytoplasmic and nuclear pattern (Fig. 1A,B). In contrast, cells treated with 5 μM Ca^{2+} ionophore A23187 display strong labelling at the nucleo/cytoplasmic boundary (Fig. 1C). A high magnification image of the nucleus reveals occasional wrinkles of the nuclear envelope (Fig. 1D), and plotting the fluorescence intensity profile of the nucleus and adjacent cytoplasm reveals a peak coinciding with the nucleo/cytoplasmic boundary in A23187-treated cells, whereas the cytoplasmic and nuclear fluorescence is reduced comparatively to control cells (Fig. 2E). In contrast to A23187 (Fig. 2B), neither bradykinin (Fig. 2C), nor thrombin nor bombesin (not shown) causes an apparent change of Anx IV immunolocalization compared with untreated cells (Fig. 2A). Furthermore, the translocation did not occur when cells were incubated, following ionophore treatment, for 1 min with Ca^{2+}-free medium before fixation (Fig. 2D). Similarly, cells treated with A23187 without external Ca^{2+} do not display any difference from control cells (not shown).

3.2. Differential effects of A23187 and bradykinin on nuclear [Ca^{2+}]

We have compared the effect of A23187 and bradykinin on intracellular [Ca^{2+}] in human fibroblasts. Using Ca^{2+} measurement by conventional microfluorimetry in Indo-1-loaded cells, bradykinin and A23187 were found to induce similar rises of intracellular [Ca^{2+}] averaged over the whole cell. To discriminate changes of intracellular [Ca^{2+}] unresolved by conventional microscopy, we have used confocal microscopy to analyze cytoplasmic and nuclear Ca^{2+} signals of cells loaded with the Ca^{2+}-indicator Fluo-3. Fig. 3B–E displays confocal images and their profile plots of cells acquired before and 2 min after stimulation. Strikingly, A23187-treated cells have a much higher signal in the nucleus than in the cytoplasm (Fig. 3C), even when basal nuclear fluorescence is lower than the cytoplasmic one (Fig. 3D). In contrast, most bradykinin-treated cells displayed a similar signal in the cytoplasm and the nucleus (Fig. 3B), and its amplitude was comparable to the cytoplasmic signal in ionophore-treated cells. In addition, when the cells were treated with A23187 without extracellular Ca^{2+}, the nuclear and cytoplasmic signals 2 min after stimulation remained close to the basal state (Fig. 3E).

To resolve changes in intracellular Ca^{2+} signals over time, we line-scanned at 2-s intervals during stimulation through the whole cell, intersecting the nucleus. Thereafter, the intensity profiles for cytoplasmic and nuclear regions of the consecutive line scans were plotted versus time. The results are shown in Fig. 4. In the cytoplasm, the kinetics and the extent of the rise of the Ca^{2+} signals are comparable for both bradykinin and

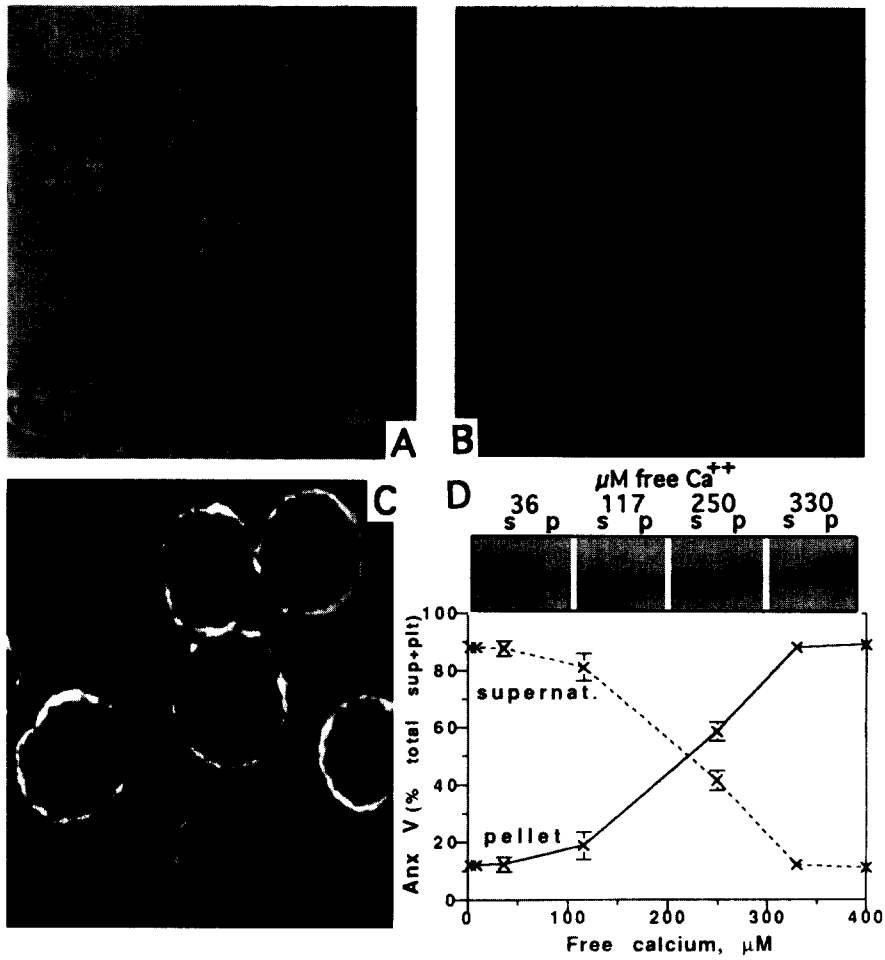

Fig. 6. Ca^{2+} dependence of Anx V binding to the nuclear membrane of isolated nuclei. (A) Phase contrast image of isolated nuclei (magnification as in B,C). (B,C) Confocal images of nuclei incubated with purified human Anx V at 2 different Ca^{2+} concentrations and processed for Anx V immunofluorescence. (B) 1 μM free Ca^{2+}; (C) 400 μM free Ca^{2+}. (D) Ca^{2+} dependence of Anx V binding to the nuclear membrane. Nuclei (p, pellet) and supernatants (s) were analyzed by Anx V immunoblotting. The top panel shows the phosphorimager picture of one immunoblot. The bottom panel displays quantitative data obtained by integration of the phosphorimager signals (mean ± S.E.M. of 3 experiments).

A23187 treatments. In contrast, in the nucleus, whereas bradykinin induces only a transient rise of Ca^{2+}, the ionophore generates a signal that remains elevated for 2 min when Ca^{2+} is present in the extracellular medium. Thus, although A23187 and bradykinin have a similar effect on $[Ca^{2+}]$ averaged over the whole cell or measured in the cytoplasm, only A23187 yields a sustained rise of nuclear $[Ca^{2+}]$.

3.3. *Anxs IV and V are specifically translocated to the nuclear membrane*

To determine the specificity of the system with respect to the annexins expressed in IMR90 cells, other annexins were also tested for Ca^{2+}-induced intracellular translocation. Similarly to Anx IV, Anx V is also translocated to the nuclear envelope by A23187 treatment (Fig. 5A,B). In contrast, A23187 does not alter the labelling of other annexins tested, including Anxs I (Fig. 5C,D), VI (Fig. 5E,F), II and VII (not shown).

3.4. *Ca^{2+}-dependent binding of Anx V to isolated nuclei*

We next studied the binding of purified Anx V to isolated rat liver nuclei obtained by centrifugation through a sucrose cushion [9]. Phase contrast microscopy indicated that the preparation was nearly 100% enriched in nuclei appearing free of any cytoskeletal extensions (Fig. 6A). These isolated nuclei were incubated with purified human Anx V at various $[Ca^{2+}]$, then processed for immunofluorescence and confocal microscopy. Anx V was found to bind to the nuclear envelope at 400 μM Ca^{2+} (Fig. 6C), but not at 1 μM Ca^{2+} (Fig. 6B). To determine the precise $[Ca^{2+}]$ requirements for this interaction, we used immunoblotting methods to measure the fraction of Anx V associated or not with the nuclear membrane in the presence of various $[Ca^{2+}]$. As shown in Fig. 6D, the association occurs at a threshold $[Ca^{2+}]$ close to 100 μM, with half-maximum binding occurring at approx. 220 μM.

4. Discussion

The main finding of this work is that Anxs IV and V translocate to the nuclear membrane during an increase of the nucleoplasmic Ca^{2+} signal. Redistribution of these annexins was observed after treatment of the cells with the Ca^{2+} ionophore A23187, which produces a profound rise of Ca^{2+} signal in the nucleus. By contrast, other agonists such as bradykinin, induce only a limited rise of nuclear Ca^{2+} which is insufficient to produce annexin translocation. In addition, we have shown

that both the stable increase in nuclear [Ca^{2+}] and the translocation of Anxs IV and V require extracellular Ca^{2+}.

Strikingly, this A23187-induced translocation, also described recently by Barwise and Walker [10], is reminiscent of that of the 85-kDa cytosolic phospholipase A_2 ($cPLA_2$). Using microscopy methods, Glover and co-workers [5] showed in the rat mast cell line RBL-2H3 that $cPLA_2$ translocated to the nuclear envelope following A23187 treatment. They suggested that this translocation might be due to a putative receptor or to a specific phospholipid composition of the nuclear membrane. However, Anxs and $cPLA_2$ constitute two different types of proteins with Ca^{2+}-regulated membrane-binding abilities. Therefore, our data imply that the translocation of Anxs and $cPLA_2$ is directly triggered by the elevation of [Ca^{2+}] in the nucleus.

Altogether, these data raise the question of how cytosolic proteins can respond to a rise in nucleoplasmic calcium. At the basal state, we have found Anxs IV and V in both the cytosol and the nucleus. Although the translocation of nuclear Anxs to the inner side of the nuclear envelope during a rise in nucleoplasmic calcium is not unexpected, our results show that even cytosolic annexins are relocalized to the nuclear membrane. Similarly, $cPLA_2$, that is excluded from the nucleoplasm in resting cells, translocates to the nuclear envelope during A23187 treatment [5]. The permeability of the nuclear envelope towards Ca^{2+} has been extensively debated for the last few years, but recent data led to the conclusion that release from Ca^{2+} stores in or around the nuclear envelope is directed into the nucleoplasm from where it can diffuse out through the permeable nuclear pore complex and thus increase the cytosolic [Ca^{2+}] in the immediate vicinity of the nuclear envelope [11].

The first Anxs described have been isolated as in vitro inhibitors of PLA_2 activity. However, it is now widely accepted that the Anxs are non-specific inhibitors of phospholipases, acting by substrate depletion (see reviews [1,3]). The physiological relevance of PLA_2 regulation by annexins in vivo is consequently questionable. Yet, the possibility that some Anxs and $cPLA_2$ colocalize during Ca^{2+} mobilization suggests that $cPLA_2$ might be actually regulated by Anxs at the nuclear membrane level. Finally, to our knowledge, the translocation described herein and by Barwise and Walker [10] constitutes the first evidence of a Ca^{2+}-regulated interaction of Anxs with cell membranes in situ, although it occurs following stimulation with a Ca^{2+} ionophore. Interestingly, relocalization of $cPLA_2$ to the nuclear envelope was initially found during A23187 treatment [4], but a partial translocation has been recently reported during IgE/antigen stimulation of mast cells [5]. It will be most interesting to determine whether Anxs are also translocated under similar stimulation.

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