Acta Biochimica Polonica

Vol. 54 No. 2/2007, 261–271

on-line at: www.actabp.pl

Regular paper

Calcium- and proton-dependent relocation of annexin A6 in Jurkat T cells stimulated for interleukin-2 secretion

Paulina Podszywalow-Bartnicka, Agnieszka Strzelecka-Kiliszek, Joanna Bandorowicz-Pikula and Slawomir Pikula✉

Department of Biochemistry, Nencki Institute of Experimental Biology, Warszawa, Poland

Received: 20 February, 2007; revised: 25 April, 2007; accepted: 21 May, 2007
available on-line: 04 June, 2007

Annexin A6 (AnxA6) is a Ca^{2+}-dependent membrane-binding protein involved in vesicular traffic. The likely participation of AnxA6 in the response of lymphocytes to Ca^{2+} signals has not been investigated yet. The present study focuses on intracellular relocation of AnxA6 in human Jurkat T lymphoblasts upon stimulation followed by transient increase of intracellular [Ca^{2+}] and exocytosis of interleukin-2 (IL-2). Stimulation of the cells under different experimental conditions (by lowering pH and/or by rising extracellular [Ca^{2+}] in the presence of ionomycin) induced time-dependent transients of intracellular [Ca^{2+}] and concomitant changes in AnxA6 intracellular localization and in IL-2 secretion, with only minor effects on cell viability and apoptosis. In resting conditions (in the presence of EGTA or with no ionophore) AnxA6 was localized uniformly in the cytosol, whereas it translocated to vesicular structures beneath the plasma membrane within 5 min following stimulation of Jurkat T cells and rise of intracellular [Ca^{2+}] at pH 7.4. Lowering the extracellular pH value from 7.4 to 6.0 significantly enhanced this process. AnxA6 changed its location from the cytosol to the secretory granules and early endosomes which seem to represent membranous targets for annexin. In conclusion, AnxA6 is sensitive to variations in intracellular [Ca^{2+}] upon stimulation of Jurkat T cells, as manifested by a switch in its intracellular localization from the cytosol to vesicular structures located in close proximity to the plasma membrane, suggestive of participation of AnxA6 in calcium- and proton-dependent secretion of cytokines by lymphocytes.

Keywords: annexin A6, calcium, ionomycin, interleukin-2, vesicular traffic, Jurkat T cells

INTRODUCTION

Cytolytic T lymphocytes use two mechanisms to kill virally infected cells, tumor cells, or even other potentially autoreactive T cells (Esser *et al.*, 1998). The first, the perforin/granule exocytosis, utilizes cytolytic granules containing cytokines that are delivered to the target cell to induce apoptosis and eventual lysis. This mechanism requires the release of Ca^{2+} from intracellular stores of the lymphocyte followed by a sustained influx of extracellular Ca^{2+}. The second mechanism, FasL/Fas-mediated cytolysis, requires *de novo* synthesis of FasL by the lymphocyte and the presence of the death receptor Fas on the target cell to induce apoptosis. In this case only a sustained influx of extracellular Ca^{2+} is required for this mechanism to be activated. Both mechanisms point to Ca^{2+} as a key factor in appropriate functioning of lymphocytes. This calls for identification of calcium-binding proteins as receptors, effectors and mediators of Ca^{2+}-dependent events, including secretion of cytokines by T lymphocytes.

Cytokines regulate cellular immune activity and are produced by a variety of cells, not only

✉**Corresponding author**: S. Pikula, Department of Biochemistry, Nencki Institute of Experimental Biology, 02-093 Warszawa, Poland; e-mail: s.pikula@nencki.gov.pl

Abbreviations: AnxA6, annexin A6; BCECF, 2′,7-bis(2-carboxymethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester; DABCO, 1,4-diazabicyclo[2.2.2]octane; IL-2, interleukin-2; DMSO, dimethylsulfoxide; PBS, phospahte-buffered saline; PI, propidium iodide; PMA, phorbol 12-myristate 13-acetate; SNARE proteins, soluble *N*-ethylmaleimide-sensitive fusion protein (NSF) attachment protein receptor proteins; TBS, Tris-buffered saline.

lymphocytes, but also monocytes and macrophages. Human cytokines are most commonly assessed at the macro level by measuring their levels in serum or plasma or in supernatants of *in vitro* stimulated blood cells. Since the process of cell stimulation often leads to programmed cell death, *in vivo* stimulation, preceding *in vitro* stimulation, is not only of potential physiological significance but may be also of practical importance in terms of interpreting the results of *in vitro* stimulation of cytokine production (Walker *et al.*, 2002). Multiparameter flow cytometry is used to examine cell-specific cytokine production after *in vitro* induction with phorbol 12-myristate 13-acetate (PMA) and ionomycin, in the presence of brefeldin A, monensin or other agents able to inhibit protein secretion.

In this context a challenging question is how intracellular Ca^{2+} transients in conjunction with signaling cascades are related to the functioning of lymphocytes, especially to their activity. Calcium-binding proteins are intracellular receptors and mediators of Ca^{2+} transients and special interest has been placed on their identification and characterization. Among the various families of calcium-binding proteins, annexins have gained special attention due to their ability to interact with cellular membranes in a dynamic and reversible manner as well as their potential activity as membrane fusogens, Ca^{2+} sensors and signal transducers in regulated exo- and endocytosis (Creutz, 1992; Donnelly & Moss, 1997; Pons *et al.*, 2001).

Among annexins, structurally related Ca^{2+}-dependent membrane-binding proteins characterized by a domain structure conferring functional diversity (Edwards & Moss, 1995; Gerke & Moss, 2002), AnxA6 is truly unique. While other annexins contain four repetitive domains, AnxA6 has eight; each repeat domain usually contains a characteristic "type 2" motif with the evolutionarily conserved sequence "GxGT–[38 residues]–D/E", which is responsible for binding of Ca^{2+} (Moss & Morgan, 2004).

At the subcellular level, AnxA6 has been localized in the cytosol, where it is found in close association with membranous structures (Golgi apparatus, vacuoles and endoplasmic reticulum), and attached to the plasma membrane (Rambotti *et al.*, 1993; Kaetzel *et al.*, 1994). Therefore, AnxA6 is considered as a cytosolic protein that exists in the cell in two forms, soluble form and a Ca^{2+}/phospholipid/cholesterol-dependent membrane-bound one (Gerke & Moss, 1997; Rescher & Gerke, 2004). In particular, changes in the intracellular distribution and concentration of cholesterol in different subcellular compartments were found to affect the reorganization of intracellular pools of Ca^{2+}-dependent and Ca^{2+}-independent AnxA6 (de Diego *et al.*, 2002). Transient annexin depletion by RNA interference and the expression of dominant-negative mutant proteins shed new light on the role of AnxA6 in membrane-related processes ranging from the control of membrane structure to certain membrane transport phenomena. Surprisingly, mice lacking AnxA6 showed no obvious phenotype related to a primary defect in vesicle docking and/or fusion events (Hawkins *et al.*, 1999; Song *et al.*, 2002). This may indicate that the lack of AnxA6 is compensated by other members of the annexin family of proteins.

AnxA6 has been implicated in a number of cellular functions, including cell growth and proliferation (Kester *et al.*, 1997), intracellular calcium homeostasis (Fleet *et al.*, 1999; Babiychuk & Draeger, 2000), ion channel activity induced by GTP (Kirilenko *et al.*, 2002) or low pH (Golczak *et al.*, 2001a; 2001b), cytoskeleton reorganization (Rescher & Gerke, 2004; Hayes *et al.*, 2004), membrane dynamics (vesicular transport and membrane fusion) during exo- and endocytosis (Kubista *et al.*, 2000; Pons *et al.*, 2001; Gerke & Moss, 2002), lysosomal targeting (Grewal *et al.*, 2000) and signal transduction pathways in which a crucial role is played by Ras proteins (Donnelly & Moss, 1998; Russo-Marie, 1999; Grewal & Enrich, 2006).

The role of AnxA6 in secretion has not been extensively investigated, but the available data suggest that AnxA6 may block vesicle SNARE protein aggregation and fusion mediated by AnxA2 and AnxA7, perhaps by competing with them for binding sites and acting as a negative regulator of exocytosis (Donnelly & Moss, 1997; Gerke *et al.*, 2005). According to this idea, AnxA6 is "switched off" in lactating breast where a strong secretory stimulus is desirable, and "switched on" in cells of the immune system where a fine-tuning mechanism of secretory control is necessary (Donnelly & Moss, 1997).

The present study focuses on a calcium- and proton-dependent sequential redistribution of AnxA6 upon ionomycin stimulation of human Jurkat T lymphoblasts to induce cytokine secretion.

MATERIALS AND METHODS

Cell culture. Human Jurkat E6.1 leukaemic T cell lymphoblasts (ECACC 88042803) were cultured in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma).

Calcium and pH treatment of the cells. Cells were incubated in a PD medium (125 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 1 mM KH_2PO_4, 10 mM glucose, 1 mM $MgCl_2$, 20 mM Hepes, pH 7.0) supplemented with 1 mM $CaCl_2$ or 5 mM EGTA (different Ca^{2+} conditions), pH 7.4 or 6.0 (different

pH conditions) for 10 min at room temperature. Intracellular [Ca^{2+}] was manipulated by incubating the cells with 5 μM ionomycin (Sigma) for 10 min at room temp. in the presence of 1 mM extracellular Ca^{2+}. Ionomycin was prepared from a stock solution in DMSO; the final concentration of DMSO did not exceed 0.05% by volume.

Immunofluorescence and confocal microscopy. Cells (10^5) were washed twice in the PD medium and incubated in the presence of 1 mM $CaCl_2$ or 5 mM EGTA, at pH 7.4 or 6.0, with or without 5 μM ionomycin for 10 min at room temp. DMSO was added as a control. The cells were immediately fixed with 3% paraformaldehyde/PD for 20 min at room temp. The fixed cells were washed in the PD medium, incubated in 50 mM NH_4Cl/PD (10 min, room temp.) and after washing permeabilized with 0.08% Triton X-100/PD (5 min, 4°C). After washing once in the PD medium, and once in Tris-buffered saline (TBS; 130 mM NaCl, 25 mM Tris/HCl, pH 7.5), the cells were incubated in a blocking solution (5% fetal bovine serum/TBS) for 45 min at room temp. Then, the cells were exposed to primary antibodies — mouse monoclonal anti-AnxA6 (Transduction Laboratories) or rabbit polyclonal anti-Rab3A, Rab5B, Rab7 (Santa Cruz Biotechnology). After 1 h incubation the cells were washed six times with 0.5% fetal bovine serum/TBS/0.05% Tween 20 (5 min each) and incubated for 1 h at room temp. with secondary antibodies — goat anti-mouse IgG conjugated with FITC (Sigma) or goat anti-rabbit IgG conjugated with AlexaFluor546 (Molecular Probes). All antibodies were prepared in 0.5% fetal bovine serum/TBS/0.05% Tween 20 and applied for 1.5 h at room temp. In control experiments, the incubation with primary antibodies was omitted. After extensive washing (four times in 0.5% fetal bovine serum/TBS/0.05% Tween 20, two times in TBS and once in H_2O) the samples were mounted in Moviol 4–88/2.5% DABCO and examined under a TCS SP2 spectral confocal and multiphoton microscope (Leica). Photographs were prepared using the Adobe-Photoshop software.

$[Ca^{2+}]_{in}$ measurements. Intracellular (cytosolic) [Ca^{2+}] was measured with the fluorescent probe Fura-2/AM (Molecular Probes) as described in (Grynkiewicz *et al.*, 1985). Five million cells were loaded with the probe by incubating in the culture medium supplemented with 1 μM Fura-2/AM for 15 min at 37°C. After washing by centrifugation the cells were suspended in the PD medium supplemented with 1 mM $CaCl_2$ in different pH conditions (7.4 or 6.0) and $[Ca^{2+}]_{in}$ was monitored fluorimetrically. The fluorescence was continuously measured at 30°C in a Shimadzu RF5000 fluorimeter set in the ratio mode using 340 nm/380 nm as excitation wavelengths and 510 nm as the emission wavelength. Time resolution of the measurements was one second. $[Ca^{2+}]_{in}$ was calibrated in each run by the addition of 10 μM ionomycin followed by the addition of 100 mM EGTA.

Intracellular pH measurements. Intracellular pH was measured with the fluorescent probe 2',7-bis(2-carboxymethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM, Molecular Probes), according to Rink and co-workers (Rink *et al.*, 1982). Five million cells were loaded with the probe by incubating in the culture medium supplemented with 1 μM BCECF/AM for 15 min at 37°C. Thereafter, the cells were washed and suspended in the PD medium supplemented with 1 mM $CaCl_2$ at different pH conditions (7.4 or 6.0) as described for $[Ca^{2+}]_{in}$ measurements. The fluorescence was measured at 30°C in a Shimadzu RF5000 fluorimeter set in the ratio mode using 500 nm (for unprotonated BCECF) and 450 (for protonated BCECF) as the excitation wavelengths and 530 nm as the emission wavelength. The assay system was calibrated by titration with NaOH or HCl in the presence of 0.003% digitonin.

Cytokine secretion assay. For intracellular cytokine analysis, 3×10^6 Jurkat T cells in 1 ml of serum-free medium were used (Sullivan *et al.*, 2000; Batliwalla *et al.*, 2000; Walker *et al.*, 2002). Cells were incubated in the presence of 1 mM $CaCl_2$ or 5 mM EGTA, at pH 7.4 or 6.0, with 0.05% DMSO or 5 μM ionomycin for 4 h at 37°C in the presence of 10 μg/ml brefeldin A (Sigma). After being washed in PBS/brefeldin A, the cells were stained for 30 min at 4°C in the dark with R-PE-conjugated mouse anti-human CD4 monoclonal antibody prepared in staining buffer (3% fetal bovine serum/0.09% sodium azide/PBS pH 7.4) according to the manufacturer's protocol (Beckton Dickinson PharMingen Int.). After washing 2 times in staining buffer the cells were fixed with 1% paraformaldehyde/PBS (20 min, room temp.), washed in PBS and permeabilized with 0.1% saponin/0.09% sodium azide/PBS (10 min, room temp.). After washing in PBS the cells were stained for 1 h at room temp. in the dark with FITC-conjugated rat anti-human IL-2 monoclonal antibody prepared in staining buffer. After washing 2 times in PBS the flow cytometry analysis of intracellular IL-2 was performed.

Cell viability and apoptosis analysis. For identification of live cells by propidium iodide (PI) staining, 10^6 cells were washed twice in the PD medium, suspended in 2 ml of PI solution (50 μg/ml PI in the PD medium) and then used directly for flow cytometry analysis. For apoptosis analysis, 3×10^6 cells were incubated in an appropriate buffer, and then in RPMI medium supplemented with 10% fetal bovine serum for 24 h. Afterwards the cells were centrifuged at $500\times g$ for 3 min and the pellet was suspended in 1 ml PBS. The cell suspension was

added into 2.5 ml of absolute EtOH (final concentration approx. 70%) and incubated over night at –20°C. Then the cells were centrifuged and suspended in 500 μl of PI solution (50 μg/ml PI, 0.1 mg/ml RNase, 0.05% Triton X-100 in PBS) for 40 min at 37°C. After that, 3 ml of PBS was added, cells were centrifuged, the supernatant was discarded while the pellet was resuspended in 1 ml PBS for flow cytometry analysis.

Flow cytometry. A FACScan flow cytometer (Becton-Dickinson) equipped with an argon laser emitting at 488 nm was used. FITC fluorescence was monitored by FL1 (570 V logarithmic) emission channel whereas PI as well as R-PE fluorescence were monitored by FL2 (470 V linear) emission channel. The number of cells (%) was counted using the CellQuest software (Becton-Dickinson) from 10^4 cells per each experimental set.

RESULTS

Ca^{2+}- and H^+-dependent relocation of AnxA6

Previous studies demonstrated Ca^{2+}- and H^+-dependent binding of annexins, such as AnxA7 and AnxA6, to biological and artificial lipid membranes (Bandorowicz *et al.*, 1992; Sen *at al.*, 1997; Clemen *et al.*, 2001; Golczak *et al.*, 2001a). In this report we followed the cellular distribution of AnxA6 protein using immunohistochemistry and fluorescence methods. For our experiments we chose Jurkat T lymphoblasts that are characterized by a high level of AnxA6 expression (Dubois *et al.*, 1995). To observe the effects of varying $[Ca^{2+}]_{in}$ and $[H^+]_{in}$ on the subcellular distribution of AnxA6, $[Ca^{2+}]$ and $[H^+]$ in Jurkat T cells were manipulated by incubating the cells for up to 10 min with or without ionomycin (added in DMSO) and in the presence of 5 mM EGTA or 1 mM $CaCl_2$ in the extracellular medium and either at pH 7.4 or pH 6.0. In control cells (in the presence of 5 mM EGTA at pH 7.4) the main pool of AnxA6 was homogenously distributed in the cytoplasm, independently of whether the cells were taken directly from the culture or after 10 min of incubation in the PD medium (Fig. 1A; see the Materials and Methods section). Lowering the extracellular pH to 6.0 in the presence of 5 mM EGTA resulted in staining of the plasma membrane (indicated by white arrows in Fig. 1B) and of the perinuclear region of the cell (indicated by yellow arrows in Fig. 1B). In the presence of 1 mM $CaCl_2$ without the ionophore at pH 7.4 also two types of AnxA6 cell localization were observed — beneath the plasma membrane (indicated by white arrows in Fig. 1C) and in the cytosol (indicated by yellow arrows in Fig. 1C). Lowering the extracellular pH to 6.0 in the presence of 1 mM $CaCl_2$ resulted in a punctate localization of AnxA6 on the plasma membrane (indicated by white arrows in Fig. 1D) and on the membranes of vesicular structures resembling endosomes or exosomes (indicated by yellow arrows in Fig. 1D).

Ionomycin treatment of lymphocytes in the presence of 1 mM $CaCl_2$ led to a relocation of the whole cell AnxA6 pool to the membrane of small vesicles localized throughout the cell as well as to the plasma membrane (Figs. 2 and 3). These changes were less pronounced at pH 7.4 although the $[Ca^{2+}]_{in}$ increased up to 6 μM within a time span of 10 min upon incubation with ionomycin in the presence of 1 mM $CaCl_2$ in the external medium (Fig. 2, lower panel). The scattered character of the Ca^{2+} fluxes at pH 7.4 in the presence of ionophore is probably related to the magnitude of Ca^{2+} oscillations accompanying the process. It is worth mentioning that cells incubated in the presence of 1 mM extracellular Ca^{2+} but without the ionophore are characterized by low $[Ca^{2+}]_{in}$, within the range of 30 to 50 nM, similar to values already reported in the literature. In the presence of ionomycin at pH 7.4 no significant differences in AnxA6 localization were observed for the first 1 min after ionomycin addition; most of the protein persisted in the cytoplasm, however, after 5–10 min, plasma membrane staining (indicated by white arrows in Fig. 2, upper panel) as well as the vesicle membrane-bound form (indicated by yellow arrows in Fig. 2, upper panel) of AnxA6 were observed.

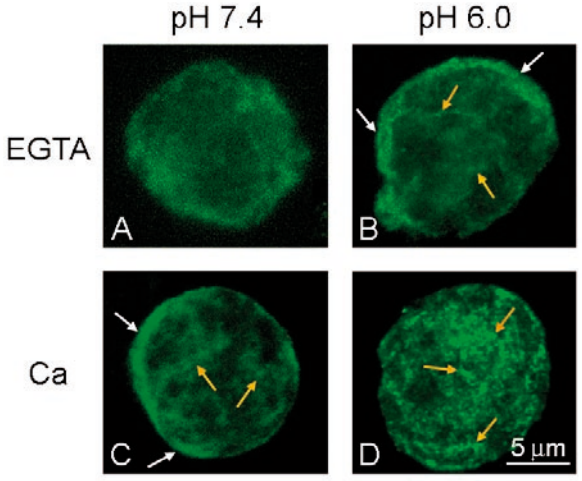

Figure 1. Localization of AnxA6 in non-stimulated Jurkat T cells.
The cells were incubated in the PD medium at pH 7.4 (A, C) or pH 6.0 (B, D), in the presence of either 5 mM EGTA (A, B) or 1 mM $CaCl_2$ (C, D). After fixation and permeabilization of the cells, localization of AnxA6 was detected by mouse anti-AnxA6 followed by goat anti-mouse-FITC staining using confocal laser scan microscopy (white arrows — plasma membrane, yellow arrows — perinuclear region or vesicles).

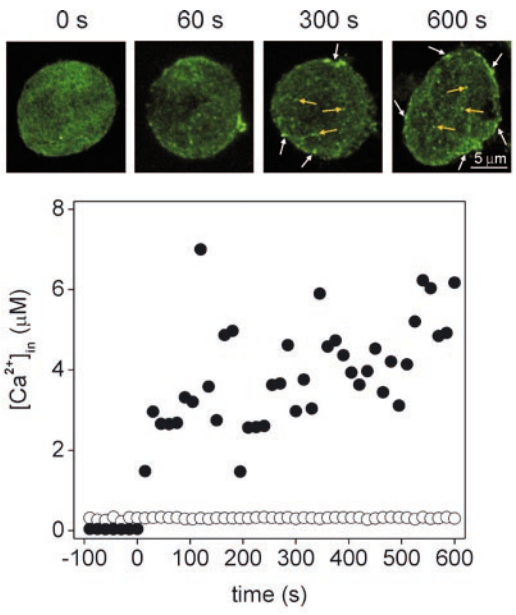

Figure 2. Ca^{2+}-dependent translocation of AnxA6 in Jurkat T cells at pH 7.4.
$[Ca^{2+}]_{in}$ was manipulated by incubating the cells in the PD medium at pH 7.4 in the presence of 1 mM $CaCl_2$. At the time indicated on the figure (upper panel) 5 µM ionomycin (or 0.05% DMSO) was added and the cells were withdrawn after 0, 60, 300 or 600 s for immediate fixation. After fixation and permeabilization, the subcellular location of AnxA6 was detected by mouse anti-AnxA6 followed by goat anti-mouse-FITC staining and confocal laser scan microscopy (white arrows — plasma membrane, yellow arrows — vesicles). Changes of $[Ca^{2+}]_{in}$ in Jurkat T cells were monitored fluorimetrically (lower panel), as described in the Materials and Methods section. DMSO (0.05%, open symbols) or 5 µM ionomycin (solid symbols) were added at time 0. One typical determination out of three is shown.

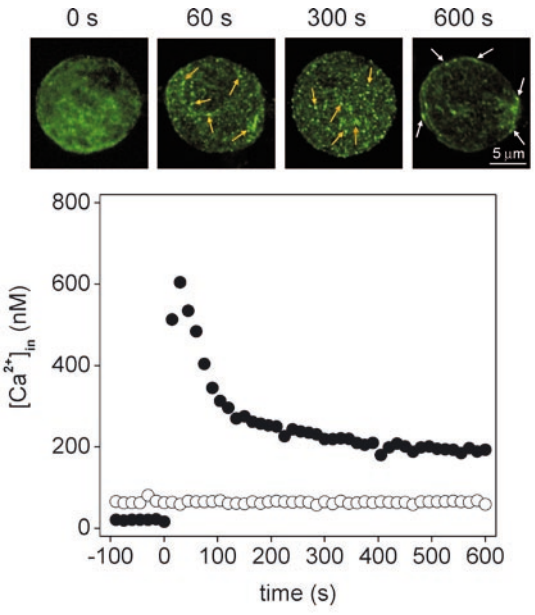

Figure 3. Ca^{2+}-dependent translocation of AnxA6 in Jurkat T cells at pH 6.0.
$[Ca^{2+}]_{in}$ was manipulated by incubating the cells in the PD medium at pH 6.0, in the presence of extracellular $CaCl_2$ (1 mM). At the time indicated on the figure (upper panel) 5 µM ionomycin (or 0.05% DMSO) was added and the cells were fixed and permeabilized. The subcellular location of AnxA6 was detected by mouse anti-AnxA6 followed by goat anti-mouse-FITC staining and confocal laser scan microscopy (white arrows — plasma membrane, yellow arrows — vesicles in perinuclear region). Changes of $[Ca^{2+}]_{in}$ in Jurkat T cells were monitored fluorimetrically (lower panel), as described in the Materials and Methods section. DMSO (0.05%, open symbols) or 5 µM ionomycin (solid symbols) were added at time 0. One typical determination out of three is shown.

To slow down the calcium uptake by Jurkat T cells in the presence of ionomycin and 1 mM external $CaCl_2$ we lowered the pH of the external medium to 6.0. Under these conditions, between 1 to 5 min after addition of the ionophore AnxA6 accumulated in association with membranes of vesicular structures in the perinuclear region of the cell (indicated by yellow arrows in Fig. 3, upper panel). After 10 min AnxA6 finally relocated to the plasma membrane (indicated by white arrows in Fig. 3, upper panel). These changes were preceded at pH 6.0 by changes in $[Ca^{2+}]_{in}$ reaching the maximum of 544.08±114.44 nM within 30 s after addition of the ionophore and then transiently decreasing within 10 min to a new resting $[Ca^{2+}]_{in}$ of 182.89±13.03 nM (Fig. 3, lower panel). Lowering the extracellular pH from 7.4 to 6.0 in the absence of ionophore did not affect $[Ca^{2+}]_{in}$, while the intracellular pH dropped from 6.92±0.01 to 6.38±0.04. It is worth to note that addition of DMSO to the cells did not influence $[Ca^{2+}]_{in}$ at any pH values, and the spectral properties of Fura-2/AM were not affected at low pH.

Our immunohistochemistry and fluorescence results presented above suggest that AnxA6 relocates to the membranous structures of the cell upon stimulation. To identify the type of membranes to which AnxA6 preferentially binds we examined the localization of AnxA6 in comparison with markers of various vesicular structures in control and stimulated Jurkat T cells (Fig. 4). In cells treated with EGTA AnxA6 staining did not change with pH and the protein did not co-localize with any of the markers examined (Fig. 4A–F). Changes in intracellular $[Ca^{2+}]$ induced by incubation of the cells in the presence of 1 mM $CaCl_2$ in the extracellular medium at pH 7.4 induced AnxA6 translocation to intracellular membranes located near the plasma membrane, identified by co-localization with Rab3A as secretory granules that may participate in the first phase of regulated secretion (indicated

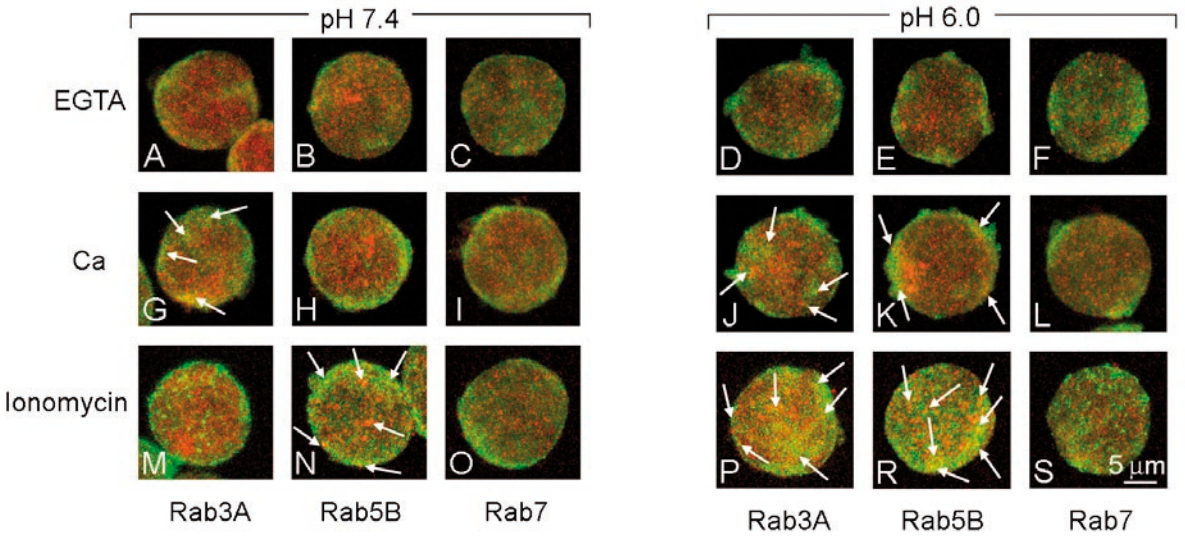

Figure 4. Ca^{2+}- and H^+-dependent translocation of AnxA6 to membranous compartments in Jurkat T cells.
The cells were incubated in the PD medium at pH 7.4 (A, B, C, G, H, I, M, N, O) or pH 6.0 (D, E, F, J, K, L, P, R, S), in the presence of either 5 mM EGTA (A, B, C, D, E, F), 1 mM $CaCl_2$ (G, H, I, J, K, L) or 1 mM $CaCl_2$ and 5 µM ionomycin (M, N, O, P, R, S). After fixation and permeabilization of the cells, localization of AnxA6 together with Rab3A (A, D, G, J, M, P), Rab5B (B, E, H, K, N, R) or Rab7 (C, F, I, L, O, S) was detected by mouse anti-AnxA6 antibody and with rabbit anti-Rab3A, Rab5B or Rab7 antibodies, followed by goat anti-mouse-FITC together with goat anti-rabbit-AlexaFluor546 staining, using confocal laser scan microscopy (arrows — protein co-localization indicated by yellow color of merged photographs).

by arrows in Fig. 4G). Mild acidification of the medium enhanced the staining of AnxA6 in exosomal membranes in the cytosol (indicated by arrows in Fig. 4J) and induced translocation of this protein beneath the plasma membrane to early endosomes identified by Rab5B staining (indicated by arrows in Fig. 4K). Upon the rise of intracellular $[Ca^{2+}]$ due to the presence of ionophore, AnxA6 was found attached to the vesicular structures identified as early endosomes by distribution of their marker protein, Rab5B (indicated by arrows in Fig. 4N). Extracellular pH 6.0 significantly enhanced this process. Thus, two populations of AnxA6 can be distinguished: one associated with early endosomes (indicated by arrows in Fig. 4R) and the other with membranous structures identified by co-localization with Rab3A as secretory granules (indicated by arrows in Fig. 4P) participating, most probably, in the second phase of regulated secretion. There was no co-staining of AnxA6 with Rab7, a marker of late endosomes, under our experimental conditions (Fig. 4F, L, S).

Ca^{2+}- and H^+-dependent secretion of interleukin-2

Measurement of cytokine production is a valuable adjunct to standard immunological assays in defining several pathological processes (Sullivan *et al.*, 2000), therefore we performed detection of intracellular cytokines using a technique in which cells are permeabilized and antibodies are used to detect cytokines by flow cytometry (Batliwalla *et al.*, 2000; Walker *et al.*, 2002). We examined the effect of changes in $[Ca^{2+}]_{in}$ induced by incubation of Jurkat T cells with or without ionomycin and in the presence of 5 mM EGTA or 1 mM $CaCl_2$ in the extracellular medium on IL-2 production by these cells (Fig. 5). The experiments were performed at pH 7.4 (filled bars) or pH 6.0 (open bars) in the presence of brefeldin A, an agent able to inhibit protein secretion, in order to determine the secretory granules containing IL-2 inside the cells. At pH 7.4 in control conditions (no further additions, Fig. 5A and B, a) or when the cells were treated with EGTA (Fig. 5A and B, b) or $CaCl_2$ (Fig. 5A and B, c) or DMSO (Fig. 5A and B, d) but without the ionophore, the majority of Jurkat T cells are able to express CD4 (not shown), while IL-2 is produced by a small number of cells (6.9±3.7% for 10 min stimulation, Fig. 5A, a–d, and 4.8±0.1% for 4 h stimulation, Fig. 5B, a–d). At pH 6.0 (Fig. 5, open bars) the production of IL-2 was slightly inhibited in control cells as compared to pH 7.4 (Fig. 5, filled bars). Ionomycin treatment in the presence of 1 mM $CaCl_2$ led to an increase in the number of cells producing IL-2 to 9.7±3.8% of the cell population after 10 min at pH 7.4 (Fig. 5A, e, filled bars) but longer stimulation up to 4 h was necessary to reach a statistically significant difference in the number of cells producing IL-2 (an increase to 42.2±10.4%, Fig. 5B, e, filled bars). The changes presented above were less pronounced at

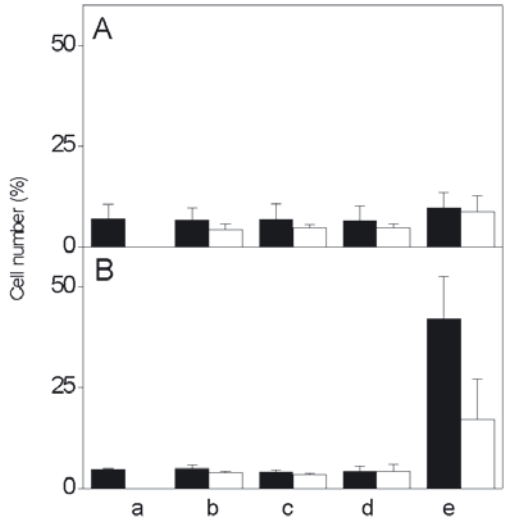

Figure 5. Ca^{2+}- and H^+-dependent cytokine secretion by Jurkat T cells.
Intracellular [Ca^{2+}] and [H^+] were manipulated by incubating the cells for 10 min (A) or 4 h (B) in the PD medium at pH 7.4 (filled bars) or 6.0 (open bars) with no further additions (a) or in the presence of 5 mM EGTA (b), 1 mM $CaCl_2$ (c), 0.05% DMSO (d) or 5 μM ionomycin and $CaCl_2$ (e) as indicated. Cells were stained with R-PE-anti-CD4, fixed, permeabilized, and then stained with FITC-anti-IL-2 (see the Materials and Methods section). Brefeldin A (10 μg/ml) was present in the media throughout the experiment. The number of cells with intracellular IL-2 (FITC) fluorescence was measured by flow cytometry. Results are expressed as means±S.E. of three experiments.

pH 6.0 when the number of cells producing IL-2 did not exceed 20% even after 4 h of stimulation (Fig. 5A and B, e, open bars). Cell activation with ionomycin caused a statistically non significant decrease in CD4 expression in the cells, as reported earlier in the literature (Andersson & Coleclough, 1993), regardless of the external pH.

Viability of Jurkat T cells under experimental conditions

Taking into consideration that conditions used during our experiments could affect cell viability, we analyzed it by PI staining. No significant differences in cell viability could be detected between the experimental variants (Table 1). Staining of fixed cells with PI revealed that 24 h after treatment of cells with 1 mM $CaCl_2$ and ionomycin for 10 min at pH 7.4, up to 32% of the cells underwent apoptosis. Treatment of the cells with ionomycin at pH 6.0 did not result in an extensive apoptosis (< 7%, Table 2).

DISCUSSION

In the present study we addressed the problem of a potential role of AnxA6 in calcium-dependent secretion of cytokines by T lymphocytes upon stimulation leading to changes in intracellular ion homeostasis. Lymphocytes have been proven to be a good model to investigate exocytosis. Two forms of exocytosis can be distinguished — constitutive, in which vesicle formation is followed by an immediate fusion, and regulated, in which vesicles may wait in the cytoplasm for a signal before fusion is initiated (Donnelly & Moss, 1997). In the latter case, the rise in calcium concentration may be restricted to localized regions immediately beneath the plasma membrane and this may prevent vesicles in the cell interior from docking and fusing with the membrane. The activation of T lymphocytes through the TCR/CD3 complex results in an enhanced induction of the autocrine growth factor IL-2, governing expansion of antigenic T cells (Nell *et al.*, 2002). Inducible expression of IL-2 is tightly regulated by multiple transcription factors that bind at distinct sites on the IL-2 promoter (Jhun *et al.*, 2005). Ionomycin and PMA are commonly used in *in vitro* experiments for

Table 1. Viability of Jurkat T cells in different experimental conditions.

The cells were incubated in the PD medium essentially as described in the legends to Figs. 1–4. Live cells were identified by PI staining and flow cytometry analysis, as described in the Materials and Methods section. The results of one typical experiment are presented.

		Live cells (%)			
pH	Time (s)	5 mM EGTA	1 mM $CaCl_2$	0.05% DMSO	5 μM inomycin
7.4	60	97.82	98.55	98.44	97.22
7.4	300	94.82	98.03	97.87	97.31
7.4	600	93.05	97.01	96.42	95.65
6.0	60	98.23	97.93	98.79	98.14
6.0	300	94.43	97.20	97.94	96.33
6.0	600	93.83	96.55	97.25	95.07

Table 2. Apoptosis in Jurkat T cells.

The cells were incubated in the PD medium for 10 min essentially as described in the legends to Figs. 1–4 and then were grown for additional 24 h. Apoptotic cells were identified by fixation, PI staining and flow cytometry analysis, as described in the Materials and Methods section. The results from two experiments are presented.

	Apoptotic cells (%)			
pH	5 mM EGTA	1 mM $CaCl_2$	0.05% DMSO	5 μM ionomycin
7.4	2.70–5.10	2.71–5.47	3.04–5.10	13.83–32.37
6.0	2.29–5.93	3.37–7.03	3.28–5.88	4.06–6.82

stimulation of Jurkat T cells. It is well established that in the presence of those agents stimulated lymphocytes secrete cytokines (Marti *et al.,* 2001; Pardo *et al.,* 2003). Our studies revealed that an increase in intracellular [Ca^{2+}] caused a major fraction of Jurkat T cells to synthesize CD4, but a smaller fraction produced IL-2, in agreement with observations made by other investigators (Batliwalla *et al.,* 2000). Therefore, we have proven that a several hour-long incubation of the cells in the presence of 1 mM $CaCl_2$ and ionophore at pH 7.4 is a useful experimental procedure to stimulate a significant number of Jurkat T cells to secrete cytokine. We are aware that in order to obtain a direct proof of the involvement of AnxA6 in cytokine secretion a quantitative determination of IL-2 using ELISA in cell cultures of control and activated T cells, with AnxA6 overexpression or silencing, would be valuable.

When we analyzed the subcellular distribution of AnxA6 in non-stimulated Jurkat T cells, we observed that the main pool of AnxA6 is localized in the cytoplasm (under these conditions, [Ca^{2+}] is strongly reduced in the presence of EGTA). Higher concentrations of Ca^{2+} and H^+ in the culture medium induced punctate staining throughout the cell, probably indicative of binding of AnxA6 to vesicular structures. An effective Ca^{2+} influx across the plasma membrane of Jurkat T cells was achieved using ionomycin (external [Ca^{2+}] = 1 mM, pH 6.0). In a time frame of 10 min, we first observed a shift of AnxA6 from the cytosol to the secreting plasma membrane (Rab3A-positive) and later from the perinuclear region to the vesicular membranes identified by us as Rab5B-positive early endosomes, indicating a Ca^{2+}- and H^+-dependent transfer of AnxA6 molecules to different membrane systems in the cell. Our results are in agreement with the most recent knowledge about AnxA6 as a very dynamic protein that seems to shuttle between the cytosol, cell surface and exo- as well as endocytic compartments (Gerke *et al.,* 2005). Elevation of $[Ca^{2+}]_{in}$ induced the translocation of cytosolic and membrane-bound AnxA6 to ordered (lipid rafts/caveolae) and disordered regions of the plasma membrane playing a modulatory role in spatiotemporal Ras/MAPK and Ras/$p120^{GAP}$ signaling (Grewal *et al.,* 2004; Grewal & Enrich, 2006). A similar behavior, e.g. Ca^{2+}- and H^+-dependent intracellular relocation from the cytosol to cellular membranes was reported for AnxA7 and occurred within 17 s (Clemen *et al.,* 2001). In the cited study, an increase in $[Ca^{2+}]_{in}$ was achieved by incubating the cells with 10 μM ionomycin in the presence of 1 mM extracellular Ca^{2+} at pH 8.2. In another set of experiments, the binding of secretory granules, their aggregation and fusion was activated in a concentration-dependent manner by AnxA7 and arachidonic acid, but only at low calcium concentrations (10 μM) and at pH 6.0 (Sen *et al.,* 1997). In cells treated with a calcium ionophore (A23187) and PMA AnxA7 binding to the membrane fraction increased by 120%, with a concomitant decrease of the cytosolic pool of the protein by 40%, in comparison to non-stimulated control cells (Chander *et al.,* 2003). All these reports suggest that upon a stimulus of certain strength annexins are able to change their intracellular localization from the cytosol to cellular membranes where they can perform specific functions likely related to the membrane dynamic processes accompanying vesicular transport.

In this report, ionomycin stimulation of Jurkat T cells and the concomitant influx of Ca^{2+} from the extracellular millieu mimicking physiological processes were achieved at pH 6.0. It could be related to the sensitivity of junctional ion channels. It has been reported that acidification of the external millieu to pH 6.3 depressed the conductance of junctional channels by directly or indirectly affecting channel proteins (Abudara *et al.,* 2001). The problematic part of the use of ionomycin is that it may induce apoptosis-like events in the cells, such as membrane blebbing, mitochondrial depolarization, externalization of phosphatidylserine and nuclear permeability changes observed within 15 min of treatment (Casas *et al.,* 2006). This could be at least partially reversed by overexpression of phospholipase A_2 or pretreating the cells with arachidonic acid (Casas *et al.,* 2006).

We suggest that AnxA6 with its well established Ca^{2+}- and H^+-dependent membrane binding property and high level of expression in Jurkat T

cells could be considered as an important molecule playing a role in secretory granule-plasma membrane fusion and/or secretion of cytokines by T lymphocytes. Our co-localization studies showed that changes in intracellular [Ca^{2+}] induced by incubation of the cells in the presence of 1 mM external $CaCl_2$ at pH 7.4 induced AnxA6 translocation to Rab3A-positive secretory granules likely participating in the first phase of regulated secretion during which docked granules are primed to be readily releasable and triggered for fusion with the plasma membrane. Under such conditions the number of cells producing IL-2 found in our flow cytometry analysis was significantly higher comparing to control cells. Performing the experiments at pH 6.0 permitted us to show that AnxA6 participates also in the second phase of regulated secretion, during which a reserve pool of store granules is supplied for docking or directly triggered for plasma membrane fusion. The number of cells producing IL-2 was not so impressive but this may be explained by the inhibitory action of AnxA6 on vesicle aggregation and fusion mediated by AnxA2 and AnxA7, as reported by other investigators (Zaks & Creutz, 1990). A role of annexins, among them AnxA6, in secretion has already been postulated and attributed to controlling intracellular calcium mobilization, to regulation of vesicular trafficking, and to inhibition of phospholipase A_2 activity and blood coagulation (Creutz, 1992; Donnelly & Moss, 1997). In addition, a link between calcium signaling, low pH compartments in exocytosis and AnxA6 was recently established on the basis of an analysis of mutant mice with reduced levels of AnxA6 (Song *et al.,* 2002). Moreover, AnxA6 was found to participate in a concentration-dependent release of Ca^{2+} from chromaffin granules (Jones *et al.,* 1994). Considering the predominant localization of AnxA6 as attached to the apical region of the plasma membrane, the observations listed above may suggest that this protein could participate in the secretory events by mediating the localized release of Ca^{2+} at contact sites between chromaffin granules and the plasma membrane. If vesicle aggregation depends on annexin-mediated contact formation between neighboring membranes, it is conceivable that AnxA6 may disrupt this interaction by competing with AnxA2 for critical binding sites. It has been proposed (Donnelly & Moss, 1997) that AnxA2 heterotetramer may form a bridge between the faces of two adjacent vesicles while AnxA6, with its unique spatial configuration, could disrupt this bridge by competing for AnxA2 binding sites on the lipid membrane. Some investigators propose a SNARE-based membrane-fusion model in which addition of some annexins and Ca^{2+} accelerate the fusion process by improving the kinetics of the initial membrane-contact formation, whereas addition of other annexins may inhibit the fusion process by shielding membrane surfaces (Gerke *et al.,* 2005). Our strategy to use ionophore as a non-physiological stimulus of lymphocytes and mild extracellular acidification was key to resolving the problem whether only the calcium- and low pH-dependent pool of AnxA6 is the candidate to be the secretory inhibitor. The phenomenon that the expression of AnxA6 in circulating T and B lymphocytes, as compared to immature cells of both lineages, correlates with a secretory phenotype confirmed that for tightly controlled processes such as secretion, proteins like AnxA6 that act as negative regulators provide the cell with a more sophisticated system of control than positive mediators alone (Donnelly & Moss, 1997).

CONCLUSIONS

In conclusion, we suggest that AnxA6 belongs to a family of vesicular traffic mediators or vesicular traffic markers that are regulated by intracellular calcium transients. In the present study, we have demonstrated that AnxA6 expressed in Jurkat T lymphoblasts responds to intracellular Ca^{2+} and H^+ transients by sequential redistribution from the cytoplasm to the vesicular structures located beneath the plasma membrane that represent either storage organelles or exosomes actually on the way to the plasma membrane to secrete cytokines upon a Ca^{2+} (and/or H^+) stimulus. Further experiments, like treatment of cells by other stimulators (PMA/ionomycin, calcium ionophore A23187, calcineurin) and agonists (adenosine analog AICAR, GSK-53, cyclosporine A) of IL-2 production, are necessary to better understand the functional role of AnxA6 isoforms in vesicular traffic in lymphocytes, especially to establish the involvement of either isoform in intracellular transport of cytokines, fusion of vesicles with the plasma membrane, and secretion.

Acknowledgements

This work was supported by grant N301 049 31/1592 from the Ministry of Science and Higher Education.

REFERENCES

Abudara V, Jiang RG, Eyzaguirre C (2001) Acidic regulation of junction channel between glomus cells in the rat carotic body. Possible role of $[Ca^{2+}]_i$. *Brain Res* **916:** 50–60.

Andersson S, Coleclough C (1993) Regulation of CD4+ and CD8+ expression on mouse T-cells. *J Immunol* **151:** 5123–5134.

Babiychuk EB, Drager A (2000) Annexins in cell membrane dynamics. Ca^{2+}-regulated association of lipid microdomains. *J Cell Biol* **150:** 1113–1124.

Bandorowicz J, Pikula S, Sobota A (1992) Annexins IV (p32) and VI (p68) interact with erythrocyte membrane in a calcium-dependent manner. *Biochim Biophys Acta* **1105:** 201–206.

Batliwalla FM, Rufer N, Lansdorp PM, Gregersen PK (2000) Oligoclonal expansion in the CD8+CD28– T cells largely explain the shorter telomeres detected in this subset: analysis by flow FISH. *Human Immunol* **61:** 951–958.

Casas J, Gijon MA, Vigo AG, Crespo MS, Balsinde J, Balboa MA (2006) Overexpression of cytosolic group IVA phospholipase A2 protects cells from Ca^{2+}-dependent death. *J Biol Chem* **281:** 6106–6116.

Chander A, Sen N, Naidu DG, Spitzer AR (2003) Calcium ionophore and phorbol ester increase membrane binding of annexin A7 in alveolar type II cells. *Cell Calcium* **33:** 11–17.

Clemen CS, Herr C, Lie AA, Noegel AA, Schröder R (2001) Annexin VII: an astrogial protein exhibiting a Ca^{2+}-dependent subcellular distribution. *NeuroReport* **12:** 1139–1144.

Creutz CE (1992) The annexins and exocytosis. *Science* **258:** 924–951.

de Diego I, Schwartz F, Siegfried H, Dauterstedt P, Heeren J, Beisiegel U, Enrich C, Grewal T (2002) Cholesterol modulates the membrane binding and intracellular distribution of annexin 6. *J Biol Chem* **277:** 32187–32194.

Donnelly SR, Moss SE (1997) Annexins in the secretory pathway. *Cell Mol Life Sci* **53:** 533–538.

Donnelly SR, Moss SE (1998) Functional analysis of the human annexin I and VI gene promoters. *Biochem J* **332:** 681–687.

Dubois T, Soula M, Moss SE, Russo-Marie F, Rothhut B (1995) Potential interaction between annexin VI and a 56-kDa protein kinase in T cells. *Biochem Biophys Res Commun* **212:** 270–278.

Edwards HC, Moss SE (1995) Functional and genetic analysis of annexin VI. *Mol Cell Biochem* **149/150:** 293–299.

Esser MT, Haverstick DM, Fuller CL, Gullo CA, Braciale VL (1998) Ca^{2+} signaling modulates cytolytic T lymphocyte effector functions. *J Exp Med* **187:** 1057–1067.

Fleet A, Ashworth R, Kubista H, Edwards H, Bolsover S, Mobbs P, Moss SE (1999) Inhibition of EGF-dependent calcium influx by annexin VI is splice form-specific. *Biochem Biophys Res Commun* **260:** 540–546.

Gerke V, Moss SE (1997) Annexins and membrane dynamics. *Biochim Biophys Acta* **1357:** 129–154.

Gerke V, Moss SE (2002) Annexins: from structure to function. *Physiol Rev* **82:** 331–371.

Gerke V, Creutz CE, Moss SE (2005) Annexins: linking Ca^{2+} signalling to membrane dynamics. *Nat Rev Mol Cell Biol* **6:** 449–461.

Golczak M, Kicinska A, Bandorowicz-Pikula J, Buchet R, Szewczyk A, Pikula S (2001a) Acidic pH-induced folding of annexin VI is a prerequisite for its insertion into lipid bilayers and formation of ion channels by the protein molecule. *FASEB J* **15:** 1083–1085.

Golczak M, Kirilenko A, Bandorowicz-Pikula J, Pikula S (2001b) Conformational states of annexin VI in solution induced by acidic pH. *FEBS Lett* **496:** 49–54.

Grewal T, Enrich C (2006) Molecular mechanisms involved in Ras inactivation: the annexin A6–$p120^{GAP}$ complex. *BioEssays* **28:** 1211–1220.

Grewal T, Heeren J, Mewawala D, Schnitgerhans T, Wendt D, Salomon G, Enrich C, Beisiegel U, Jackle S (2000) Annexin VI stimulates endocytosis and is involved in the trafficking of low density lipoprotein to the prelysosomal compartment. *J Biol Chem* **275:** 33806–33813.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450.

Hawkins TE, Roes D, Monkhouse J, Moss SE (1999) Immunological development and cardiovascular function are normal in annexin VI null mutant mice. *Mol Cell Biol* **19:** 8028–8032.

Hayes MJ, Rescher U, Gerke V, Moss SE (2004) Annexin-actin interactions. *Traffic* **5:** 571–576.

Jhun BS, Oh YT, Lee JY, Kong Y, Yoon K-S, Kim SS, Baik HH, Ha J, Kang I (2005) AICAR suppresses IL-2 expression through inhibition of GSK-3 phosphorylation and NF-AT activation in Jurkat T cells. *Biochem Biophys Res Commun* **332:** 339–346.

Jones PG, Fitzpatrick S, Waismann DM (1994) Chromaffin granules release calcium on contact with annexin VI: implications for exocytosis. *Biochemistry* **33:** 8180–8187.

Kaetzel MA, Pula G, Campos B, Uhrin P, Horseman N, Dedman JR (1994) Annexin VI isoforms are differentially expressed in mammalian tissues. *Biochim Biophys Acta* **1223:** 368–374.

Kester HA, van der Leede BM, van der Saag PT, van der Burg B (1997) Novel progesterone target genes identified by an improved differential display technique suggest that progestin-induced growth inhibition of breast cancer cells coincides with enhancement of differentiation. *J Biol Chem* **272:** 16637–16643.

Kirilenko A, Golczak M, Pikula S, Buchet R, Bandorowicz-Pikula J (2002) GTP-induced membrane binding and ion channel activity of annexin VI: is annexin VI a GTP biosensor? *Biophys J* **82:** 2737–2745.

Kubista H, Sacre S, Moss SE (2000) Annexins and membrane fusion. *Subcell Biochim* **34:** 73–131.

Marti F, Krause A, Post NH, Lyddane C, Dupont B, Sadelain M, King PD (2001) Negative-feedback regulation of CD28 costimulation by a novel mitogen-activated protein kinase phosphatase, MKP6. *J Immunol* **166:** 197–206.

Moss SE, Morgan RO (2004) The annexins. *Genome Biology* **5:** 219–226.

Nell AE (2002) T-cell activation through the antygen receptor. Part 2: signaling components, signaling pathways, and signal integration at the T-cell antygen receptor synapse. *J Allergy Clin Immunol* **109:** 758–770.

Pardo J, Buferne M, Martinez-Lorenzo MJ, Naval J, Schmitt-Verhulst AM, Boyer C, Anel A (2003) Differential implication of protein kinase C isoforms in cytotoxic T lymphocyte degranulation and TCR-induced Fas ligand expression. *Int Immunol* **15:** 1441–1450.

Pons M, Grewal T, Rius E, Schnitgerhans T, Jackle S, Enrich C (2001) Evidence for the involvement of annexin 6 in the trafficking between the andocytic compartment and lysosomes. *Exp Cell Res* **269:** 13–22.

Rambotti MG, Spreca A, Donato R (1993) Immunocytochemical localization of annexins V and VI in human placentae of different gestational ages. *Cell Mol Biol Res* **39:** 579–588.

Rescher U, Gerke V (2004) Annexins – unique membrane binding proteins with diverse functions. *J Cell Sci* **117:** 2631–2639.

Rink TJ, Tsien RY, Pozzan T (1982) Cytoplasmic pH and free Mg^{2+} in lymphocytes. *J Cell Biol* **95:** 189–196.

Russo-Marie F (1999) Annexin V and phospholipid metabolism. *Clin Chem Lab Med* **37:** 287–291.

Sen N, Spitzer AR, Chander A (1997) Calcium-dependence of synexin binding may determine aggregation and fusion of lamellar bodies. *Biochem J* **322:** 103–109.

Song G, Harding SE, Duchen MR, Tunwell R, O'Gara P, Hawkins TE, Moss SE (2002) Altered mechanical properties and intracellular calcium signaling in cardiomyocytes from annexin 6 null-mutant mice. *FASEB J* **16:** 622–624.

Sullivan KE, Cutilli J, Piliero LM, Ghavimi-Alagha D, Starr SE, Campbell DE, Douglas SD (2000) Measurement of cytokine secretion, intracellular protein expression, and mRNA in resting and stimulated peripheral blood mononuclear cells. *Clin Diag Lab Immunol* **7:** 920–924.

Walker D, Jason J, Wallance K, Slaughter J, Whatley V, Han A, Nwanyanwu OC, Kazembe PN, Dobbie H, Archibald L, Jarvis WR (2002) Spontaneous cytokine production and its effect on induced production. *Clin Diag Lab Immunol* **9:** 1049–1056.

Zaks WJ, Creutz CE (1990) Annexin-chromaffin granule membrane interactions: a comparative study of synexin, p37 and p67. *Biochim Biophys Acta* **1020:** 149–160.