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Annexins sense changes in intracellular pH during hypoxia

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The pH_i (intracellular pH) is an important physiological parameter which is altered during hypoxia and ischaemia, pathological conditions accompanied by a dramatic decrease in pH_i. Sensors of pH_i include ion transport systems which control intracellular Ca^{2+} gradients and link changes in pH_i to functions as diverse as proliferation and apoptosis. The annexins are a protein family characterized by Ca^{2+}-dependent interactions with cellular membranes. Additionally, *in vitro* evidence points to the existence of pH-dependent, Ca^{2+}-independent membrane association of several annexins. We show that hypoxia promotes the interaction of the recombinant annexin A2–S100A10 (p11) and annexin A6 with the plasma membrane. We have investigated *in vivo* the influence of the pH_i on the membrane association of human annexins A1, A2, A4, A5 and A6 tagged with fluorescent proteins, and characterized this interaction for endogenous annexins present in smooth muscle and HEK (human embryonic kidney)-293 cells biochemically and by immunofluorescence microscopy. Our results show that annexin A6 and the heterotetramer A2–S100A10 (but not annexins A1, A4 and A5) interact independently of Ca^{2+} with the plasma membrane at pH 6.2 and 6.6. The dimerization of annexin A2 within the annexin A2–S100A10 complex is essential for the pH-dependent membrane interaction at this pH range. The pH-induced membrane binding of annexins A6 and A2–S100A10 might have consequences for their functions as membrane organizers and channel modulators.

Key words: annexin, calcium, hypoxia, membrane interaction, pH.

INTRODUCTION

Biological systems are finely tuned and optimized to function within tightly constrained pH ranges. Changes in cytoplasmic H^+ concentration control the activities of many enzymes, transporters and ion channels, and influence such processes as muscle contraction, post-translational processing of secretory proteins, cleavage of pro-hormones [1] and ultimately cell survival and apoptosis [2]. Cellular pH homoeostasis and pH_i (intracellular pH)-dependent control of cellular functions involve pH_i sensors, and cells have developed multiple parallel mechanisms for pH regulation. The main acid/base transporters of mammalian cells are vacuolar H^+-ATPases, Na^+/H^+ exchangers, lactate$^-$/H^+ symporters and HCO_3^- transporters [3]. This redundancy underlines the importance of pH homoeostasis for cellular function.

pH_i values, which in the resting cells are in the neutral range (7.0–7.2), can change rapidly during muscle contraction: intramuscular pH declines from 7.1 to 6.5 due to skeletal muscle fatigue [4]. Hypoxia and ischaemia are accompanied by a dramatic drop of pH_i. This decrease probably results from an immediate blockade of oxidative phosphorylation and activation of glycolysis [2]. In cancer, the expansion of tumours progressively distances the cells from the vasculature, and thus from oxygen and nutrients. The pH_i of tumours becomes acidic as a result of lactate and carbonic acid accumulation, and the cells counteract this by activation of acid extrusion mechanisms, such as the growth factor and amiloride-sensitive Na^+/H^+ exchanger and carbonic anhydrase, which converts CO_2 into carbonic acid. Uptake of carbonic acid contributes to the intracellular alkalinization, critical for cancer cell survival, and ensures an acidic extracellular milieu typical of the malignant phenotype [5]. Importantly, the expression of many proteins responsible for pH homoeostasis in tumour cells is controlled by HIFs (hypoxia-inducible factors), therefore the new approaches in cancer therapy target tumour metabolism and pH_i-control systems [6].

Ca^{2+} is an important co-mediator of pH-induced effects in the cells. Cellular pH_i sensors include a variety of ion transport systems, which control intracellular Ca^{2+} gradients and might link changes in pH_i to cellular functions, including long-term effects such as cell proliferation and apoptosis. The annexins are a multigene family of Ca^{2+}- and charged phospholipid-binding proteins, which have been implicated in numerous Ca^{2+}-regulated processes inside the cell. Their carboxy-terminal cores are evolutionarily conserved and contain Ca^{2+}-binding sites, whereas their amino-terminal tails are unique and enable the proteins to interact with distinct cytoplasmic partners. Eukaryotic cells express several annexins which are involved in the regulation of multiple functions within a single cell type [7]. Previously, using biochemical methods and imaging in live cells, we have shown that four different annexins target membrane sites of distinct lipid composition, each at a different $[Ca^{2+}]_i$ (intracellular calcium concentration) [8,9]. Given the fact that several annexins are present within any one cell, it is likely that they form a sophisticated $[Ca^{2+}]_i$ sensing system, with a regulatory influence on other signalling pathways.

Parallel to these observations, there is evidence of Ca^{2+}-independent membrane association of several annexins [10–12] that points to the existence of pH-dependent binding mechanisms, most likely mediated by hydrophobic interactions. Isas et al. [13] report that human annexin A5 and hydra annexin XII are able to bind phospholipid vesicles *in vitro* at pH 5.3 and 5.8 respectively. Similar interactions were described for hydra annexin B12 [11,14] and human annexin A6 [10,15]. It should be noted that these studies were performed *in vitro* at pH values ranging from 4 to 6,

Abbreviations used: BAPTA/AM, [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester); $[Ca^{2+}]_i$, intracellular calcium concentration; CFP, cyan fluorescent protein; GFP, green fluorescent protein; ER, endoplasmic reticulum; HEK-293 cells, human embryonic kidney 293 cells; HIF, hypoxia-inducible factor; pH_i, intracellular pH; pH_o, extracellular pH; SNARF-4F/AM, seminaphtharhodafluor-4F acetoxymethyl ester; YFP, yellow fluorescent protein.

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i.e. under conditions which rarely occur in living cells. However, the observations that annexin A6 interacts with membranes of late endosomes [16] and annexin A5 induces pH-dependent vesicle aggregation and fusion [17] point to the fact that the local acidic microenvironment of certain intracellular compartments allows these Ca^{+}-independent membrane interactions to occur *in vivo*.

We investigated the influence of a hypoxia-induced decrease of pH_i on the membrane binding of fluorescent protein-labelled human annexins. We studied the pH-dependent localization of annexins A1, A2, A4, A5 and A6 in living cells and characterized their membrane interaction biochemically in a microsome binding assay. Here we demonstrate for the first time that annexin A6 and tetrameric A2–S100A10 (p11) interact with plasma membranes of living cells upon intracellular acidification independently of the $[Ca^{2+}]_i$ and that dimerization of annexin A2 within the annexin A2–S100A10 heterotetramer is essential for pH-dependent membrane interaction. Submitting the cultures to hypoxic conditions caused intracellular acidification and consequently promoted the interaction of fluorescently-labelled annexin A2–S100A10 and A6 with the plasma membrane.

EXPERIMENTAL

Reagents and antibodies

For Western blotting and immunofluorescence, monoclonal antibodies against annexins and S100A10 were purchased from Transduction Laboratories and Santa Cruz Biotechnology. Anti-GFP (green fluorescent protein) monoclonal antibody was from Clontech. Chicken anti HIF-1α antibody was a gift from Prof. M. Gassmann (University of Zurich, Zurich, Switzerland) and rabbit polyclonal anti-Sp1 antibodies were from Santa Cruz. Unless otherwise indicated, routine chemicals were from Sigma. Restriction endonucleases, Taq polymerase and T4 DNA ligase were purchased from New England Biolabs. Fluo-3/AM (Fluo-3 acetoxymethyl ester) carboxy SNARF-4F/AM (seminaphtharhodafluor-4F acetoxymethyl ester), nigericin and BAPTA/AM [1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetra-acetic acid tetrakis(acetoxymethyl ester)] were from Molecular Probes.

Tissue preparation and immunofluorescence

Human urinary bladder smooth muscle tissue was prepared as described previously [18]. For treatment at low pH, the solution was adjusted to pH 5.0 and 100 μM BAPTA. The muscle was fixed in 4 % paraformaldehyde (at pH 7.4 or 5.0) and processed as described previously [19]. For the visualization of endogenous annexins, HEK (human embryonic kidney) 293 cells were seeded onto glass coverslips, washed with Ca^{2+}-free Na^{+}-Tyrode's buffer, incubated at pH 7.4 or pH 5 for 15 min, fixed and stained with antibodies as described previously [20].

Expression of annexins as fusions with fluorescent proteins

The coding sequences of annexins A1, A2, A4, A5 and A6 as well as S100A10 were cloned into the Living Colors Fluorescent protein vectors pEGFP-N1, pECFP-N1 and pEYFP-N1 (where CFP, cyan fluorescent protein and YFP, yellow fluorescent protein) (Clontech) following the PCR as described previously [9]. A2core-YFP fusion protein was amplified from the full-length annexin A2 sequence using the forward primer 5′-ATAAA-GCTAGCATGTTTGATGCTGAGCGGGATGC-3′ corresponding to nt 145 of the annexin A2 cDNA and containing NheI cloning site (underlined); and the reverse primer 5′-ATAAACTCGAG-CCAGATCTGTCATCTCCACCACACAGGTA-3′ containing BglII and XhoI cloning sites (underlined) for insertion into pEYFP-N1, digested with NheI and XhoI. For tandem attachment of the additional copies of the A2 core sequence, the core was amplified using the same reverse primer, and a forward primer containing a BamHI site, and the PCR product inserted into pEYFP-A2core vector, digested with BglII and XhoI. Dimerization of the A4 sequence was performed similarly using a pECFP-N1 vector. Following PCR amplification of the A4 coding sequence using the forward primer 5′-AAATAAGATCT-CATGGCCATGGCAACCAAAG-3′ and the reverse primer 5′-TAAATAAGCTTATCATCTCCTCCACAGAGAACA-3′, it was inserted in-frame into the pEN1A4-CFP vector digested with BglII and HindIII (restriction sites underlined).

Cell culture and transfections

HEK293 cells were maintained in DMEM (Dulbecco's modified Eagle's medium), and HepG2 (human hepatocellular carcinoma) cells in RPMI medium, containing 2 mM glutamine, 100 units penicillin/ml, 100 μg streptomycin/ml and 10 % foetal calf serum. All cells were grown in 5 % CO_2 at 37 °C in a humidified incubator. HEK 293 and HepG2 cells were transiently transfected with plasmids using electroporation (BioRad) and analysed after incubating at 37 °C for 48 h.

Live cell imaging and intracellular calcium measurements

Live cell imaging and calcium imaging recordings were performed as described in [9]. Cells were observed under an Axiovert 200 M microscope with laser scanning module LSM 510 META (Zeiss) using a ×63 oil immersion lens. Images were acquired at a scanning speed of 6 s, with 4 s intervals between frames. Images of the Fluo-3 loaded cells were recorded and analysed using the Physiology evaluation software package (Zeiss). An average of three independent experiments is presented.

pH_i measurements

For pH_i measurements, cells were loaded with 6 μM carboxy SNARF-4F/AM (Molecular Probes) for 45 min at room temperature. Cells were excited at 543 nm and dual wavelength measurements (587 and 650 nm) were performed on a laser scanning confocal microscope (LSM 510 META, Zeiss, Germany) with intervals of 10 seconds for 5 frames. Calibration of the carboxy SNARF-4F signal was performed by the method of Thomas et al. [21] using nigericin (20 μM) in high K^{+} salines. The ratios at saturating acidic and basic conditions (pH 2.6 and 9.68 respectively) were determined, and calibration carried out using the equation

$$\mathrm{pH} = \mathrm{p}K_a - \log[(R - R_B)/(R_A - R)] \times [F_{B(\lambda 2)}/F_{A(\lambda 2)}]$$

where R is the ratio $F_{\lambda 1}/F_{\lambda 2}$ of fluorescence intensities (F) measured at the two wavelengths λl and λ2 and the subscripts A and B represent the limiting values at the acidic and basic endpoints of the titration respectively, following the manufacturer's protocol (Molecular Probes). pK_a for carboxy SNARF-4F was 6.4, and the calibration procedure yielded a R_B at pH 9.68 of 0.2134, a R_A at pH 2.6 of 1.4791 and a $F_{B(\lambda 2)}/F_{A(\lambda 2)}$ of 171.215/77.97.

Isolation of smooth muscle microsomes and HEK 293 membranes; SDS/PAGE and Western blotting analysis; protein aggregation test

Unless otherwise stated, all procedures were performed at 4 °C or on ice. Smooth muscle microsomal membranes were isolated from porcine stomach smooth muscle according to the protocol described in [9,18]. Membrane preparation from HEK 293 cells was performed using a detergent-free protocol described

in [22]. For membrane binding assays, the membranes were pelleted by centrifugation (10 min at 12000 ***g***), resuspended in a buffer containing 100 mM Mes, 100 mM EGTA, 100 mM KCl at different pH (from 5.0 to 7.0, in 0.2 pH unit increments) and incubated at 4 °C for 30 min. Membrane-bound annexins were then separated from the unbound proteins by centrifugation (20 min, 12000 ***g*** at 4 °C). The membrane-bound proteins in the pellet and the soluble proteins in the supernatant were analysed by SDS/PAGE followed by Western blotting with anti-annexin antibodies. Image analysis to estimate the protein content of the individual bands following SDS/PAGE and Western blotting was performed using PowerLook 1120 scanner and ImageQuant TL (v2003) software from Amersham Biosciences. In order to test for pH-dependent protein aggregation, microsomes were stripped of annexins by incubating them in buffer containing 100 mM EGTA at pH 7.0, and the soluble annexins were separated from the membranes by centrifugation. The pH of the resulting supernatants was adjusted to the same values as in the binding experiments (ranging from 2.0 to 7.0, in 0.2 pH unit increments), and following incubation at 4 °C, insoluble protein aggregates were pelleted by centrifugation.

Induction of hypoxia in the cultured cells and HIF-1α detection

Cells were incubated under either normoxic conditions (20 % O_2), using a standard CO_2 incubator, or hypoxic conditions (1.5 % O_2), using a hypoxia workstation (Ruskinn Technology) for 1, 6, and 24 h. Induction of HIF-1α was detected in nuclear-enriched protein extracts by Western blotting as described [23]. Nuclear-enriched proteins were separated by SDS/PAGE (7.5 % gel) and analysed by Western blotting with anti-HIF-1α antibody. The membranes were re-incubated with an anti-Sp1 antibody to control for equal loading. To localize the fluorescent protein-tagged annexins following hypoxia, the coverslips were fixed with cold ethanol inside the hypoxia workstation. To induce hypoxia during live cell imaging, cells were grown on coverslips, placed in Na^+-Tyrode's buffer ($pH_o = 7.4$, where pH_o is extracellular pH) in the recording chamber (LaCon) on a heated stage and sealed from the outside air using a lid. Oxygen in the chamber was then substituted by a mixture of air and N_2 (1.5 % O_2, 98.5 % N_2) created using a Digamix 2M 302/a-F pump (H. Woesthoff GmbH, Germany) at a constant flow rate of 3.7 litre/min.

RESULTS

Acidification of pH_o promotes a decrease of pH_i and induces translocation of annexin A6 to the plasma membrane

In order to visualize the interactions of annexins with the plasma membrane of live cells, we expressed them in HEK 293 cells as fusions with fluorescent proteins and tracked their translocation in response to various stimuli. HEK 293 cells were co-transfected with annexin A2-CFP, and annexin A6-YFP, and the cells were exposed to acidic environment ($pH_o = 3.0$) for a period of several minutes. A series of confocal images (frames) was taken every 4 s at a scanning speed of 6 s (Figure 1A and Video 1 at http://www.BiochemJ.org/bj/409/bj4090065add.htm). In non-stimulated cells (200 s), both annexins show a diffuse, predominantly cytoplasmic localization. Approx. 3 min after reducing the pH_o from 7.4 to 3, a translocation of annexin A6 to the plasma membrane was observed (350 s), and this association remained until the end of the experiment (500 s). Surprisingly, the localization of annexin A2 did not change under these conditions, which allowed the assumption that the process was Ca^{2+}-independent, since the Ca^{2+}-sensitivity of annexin A2 exceeds that of annexin A6 [9]. Therefore, in a parallel experiment,

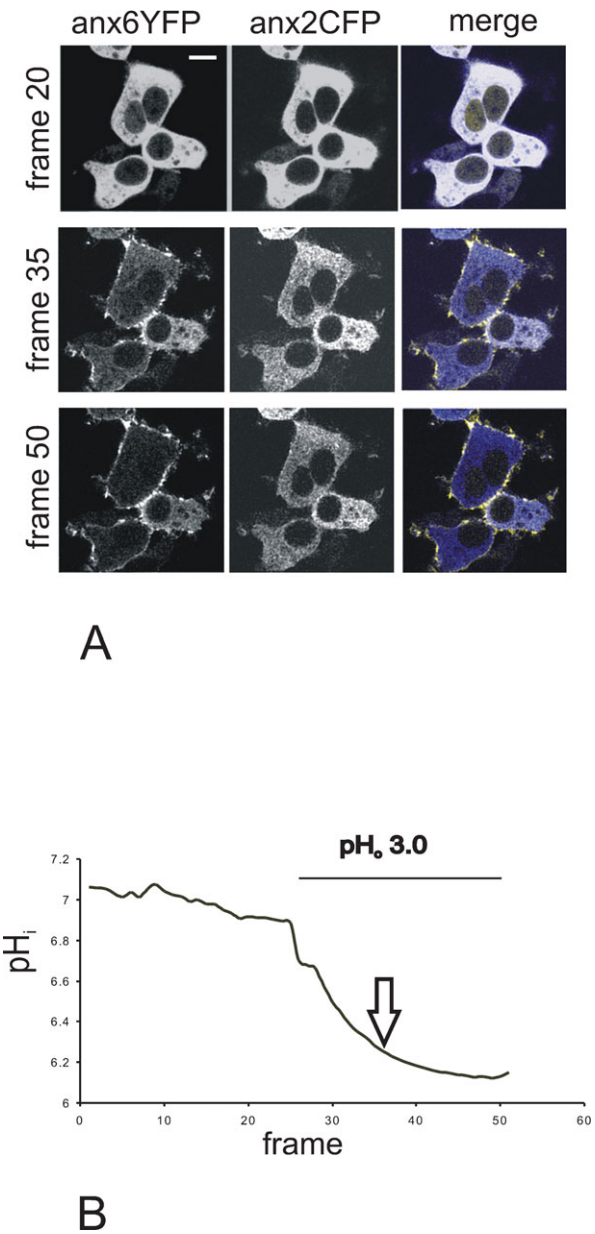

Figure 1 Extracellular acidification leads to a decrease in pH_i and the translocation of annexin A6 to the plasma membrane in HEK 293 cells

(**A**) Translocation of recombinant annexin A6 to the plasma membrane was monitored in HEK 293 cells transiently expressing annexin A6-YFP and annexin A2-CFP. 48 h post transfection, the cells were placed into a recording chamber in Ca^{2+}-free Na^+-Tyrode's buffer and time series of fluorescent images were recorded with a confocal microscope. After 250 s the coverslips were exposed to Na^+-Tyrode's buffer, pH 3.0. Annexin A6-YFP translocation to the membrane began at 350 s and persisted until the end of the experiment (500 s). Scale bar = 10 μm. See also Video1.mov (**B**) Changes in pH_i were monitored in HEK 293 cells loaded with 6 μM carboxy SNARF-4F/AM and exposed to a pH_o of 3.0. Fluorescence ratios were converted into pH values. Microscopic acquisition of results was identical to (**A**). The pH_i required for the membrane association of Annexin A6-YFP is indicated by an arrow. Data from a representative experiment are shown ($n = 3$).

we investigated the effects of extracellular acidification on the pH_i using the cell-permeable fluorescent pH indicator carboxy SNARF-4F (Figure 1B). The cells were subjected to a pH_o of 3.0, the fluorescence emission ratios measured and the results converted into pH values following calibration. In cells exposed to an acidic milieu, the pH_i gradually dropped from approx. 7.1 to 6.1 within 5 min, probably due to the decreased rate of acid extrusion

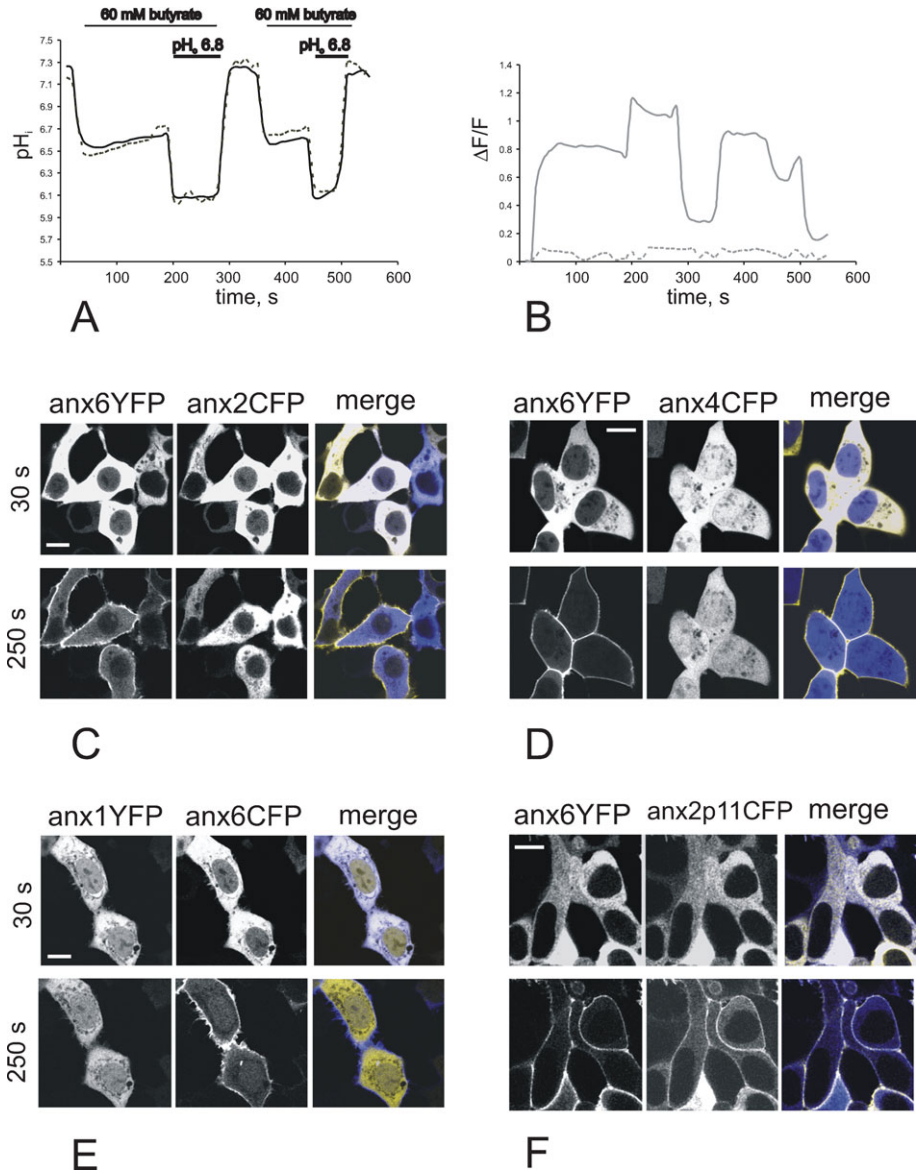

Figure 2 Effects of Na^+-butyrate and extracellular acidification on pH_i, $[Ca^{2+}]_i$ and localization of annexins in HEK 293 cells

Changes in pH_i (**A**) and $[Ca^{2+}]_i$ (**B**) were monitored simultaneously in HEK 293 cells loaded either with 3 μM Fluo-3/AM and 6 μM carboxy SNARF-4F/AM (control) or with 3 μM Fluo-3/AM, 6 μM carboxy SNARF-4F/AM and 20 μM BAPTA/AM. Responses of control cells are shown as a solid line and 20 μM BAPTA-loaded cells as a dotted line. The cells were perfused with Ca^{2+}-free Na^+-Tyrode's buffer, pH 7.4. After 30 s, the buffer was replaced by Na^+-Tyrode's containing 60 mM sodium butyrate, pH_o 7.4 and the decrease in pH_i and the increase in $[Ca^{2+}]_i$ were monitored up to 200 s when pH_o was decreased to 6.8. After buffer washes (300 s), the substances were re-applied in the same order (350 s), followed by a wash at the end of the experiment. The results of two representative experiments are shown. (**C, D, E** and **F**) HEK 293 cells co-expressing fluorescently-tagged annexins were loaded with 20 μM BAPTA/AM. Cells are shown after the application of 60 mM butyrate (30 s) and following a reduction of the pH_o to 6.8 (250 s). (**C**) Co-expression of annexin A6-YFP and annexin A2-CFP (stills from Video2.mov), (**D**) annexin A6-YFP and annexin A4-CFP (stills from Video3.mov), (**E**) annexin A6-CFP and annexin A1 YFP (stills from Video4.mov) and (**F**) annexin A6-YFP and annexin A2-CFP and S100A10 (p11)-CFP (stills from Video5.mov). Bars = 10 μm. Data from a representative experiment are shown ($n = 3$).

by Na^+/H^+ exchangers [24], and translocation of annexin A6 to the plasma membrane occurred at values below 6.2 (Figure 1B, arrow). These results indicate that a decrease in pH_o caused the reduction of pH_i, which is in agreement with previously published results [5,25], and that the translocation of annexin A6-YFP to the plasma membrane is likely to be pH-dependent.

Intracellular acidification induces annexin translocation independent of $[Ca^{2+}]_i$

Weak acids and bases are routinely used to induce changes in pH_i without influencing pH_o [26,27]. We used 60 mM sodium butyrate (salt of a weak acid) in Na^+-Tyrode's buffer (pH 7.4) containing 80 mM NaCl to adjust the osmolarity. Exposing the carboxy SNARF-4F/AM-loaded cells to butyrate caused a rapid decrease in pH_i from approx. 7.2 to 6.7 (Figure 2A). The pH_i was further decreased by acidification of pH_o from 7.4 to 6.8, which, in the presence of butyrate, resulted in a further decrease of pH_i to 6.1. These changes were completely reversible by perfusion with standard Na^+-Tyrode's buffer at pH 7.4 (Figure 2A).

Changes in pH_i are often accompanied by an alteration of $[Ca^{2+}]_i$, where intracellular acidification correlates with a marked increase in $[Ca^{2+}]_i$ due to the altered activity of calcium-entry channels, release from intracellular stores [ER

(endoplasmic reticulum) and mitochondria] and decreased affinity to Ca^{2+}-binding proteins [26,28]. We investigated the influence of butyrate-induced intracellular acidification on $[Ca^{2+}]_i$ by simultaneously loading HEK 293 cells with Fluo-3/AM and carboxy SNARF-4F/AM and perfusing them with 60 mM Na^+-butyrate in the absence of extracellular calcium (Figure 2A and 2B). A decrease of pH_i was accompanied by an increase in $[Ca^{2+}]_i$, which was reversed when the pH_i returned to normal (Figure 2B, solid line). Annexins have the ability to bind cellular membranes in a Ca^{2+}-dependent manner, hence we investigated the role of $[Ca^{2+}]_i$ in pH-induced membrane interactions. The cells were loaded with 20 μM BAPTA/AM, a cell-permeable Ca^{2+} chelator, and again subjected to butyrate perfusion. BAPTA/AM-loaded cells showed intracellular acidosis similar to the control (Figure 2A, dotted line), however, no significant elevation of $[Ca^{2+}]_i$ was recorded (Figure 2B, dotted line).

To investigate the pH-induced membrane association of annexins, HEK 293 cells expressing annexin A6-YFP and annexin A2-CFP were loaded with 20 μM BAPTA/AM and exposed to 60 mM butyrate in Ca^{2+}-free buffer at pH_o 7.4 (Figure 2C) for 30 s, followed by a pH_o of 6.8 for 250 s. A sharp decrease of pH_i in butyrate-treated cells exposed to extracellular acidification caused a rapid translocation of annexin A6-YFP to the plasma membrane (Video 2, http://www.BiochemJ.org/bj/409/bj4090065add.htm), whereas annexin A2-CFP retained its mostly cytoplasmic distribution. When the same experiments were performed with cells co-expressing annexin A6 with either annexin A4 (Figure 2D, Video 3, http://www.BiochemJ.org/bj/409/bj4090065add.htm) or annexin A1 (Figure 2E, Video 4, http://www.BiochemJ.org/bj/409/bj4090065add.htm), only annexin A6 showed the ability to interact with the plasma membrane in a pH-dependent, Ca^+-independent manner. In contrast, annexin A2-CFP showed an inconsistent localization pattern: it was able to bind to the plasma membrane of cells expressing low amounts of the protein. Annexin A2 forms a heterotetramer with S100A10 protein *in vivo*, and it is possible that, in transiently-transfected cells, the levels of endogenous S100A10 were insufficient to complex all of the recombinant annexin A2. Therefore we co-expressed annexin A2-CFP with S100A10-CFP and annexin A6-YFP, and monitored their translocation in response to butyrate-induced intracellular acidification (Figure 2F, Video 5, http://www.BiochemJ.org/bj/409/bj4090065add.htm). Under this condition, heterotetrameric annexin A2 was comparable to annexin A6 in localization to the plasma membrane (Figure 2F, 250 s). All these experiments were performed in BAPTA-loaded cells to exclude Ca^{2+}-dependent membrane binding.

Annexins A2 and A6, but not annexins A1, A4 and A5, display pH-dependent membrane interactions *in vitro*

Next we examined binding of endogenous annexins to smooth muscle microsomes at different pH values in the absence of $[Ca^{2+}]_i$. Since the ability of EGTA to bind Ca^{2+} is affected by pH, we used the MaxChelator program Winmaxc 2.40 to determine the EGTA concentration required to keep the free Ca^{2+} concentration below 100 nM at any pH point. In agreement with our results *in vivo* (above) and previous results [10,15], annexin A6 interacted with microsomal membranes in a Ca^{2+}-independent manner (in the presence of 100 mM EGTA), with a half-maximal binding at pH 6.0 (Figure 3A). Both annexin A2 and S100A10 bound microsomes with half-maximum binding at pH 6.4, and showed an identical distribution between the soluble and membrane-bound states (Figure 3B), indicating that the heterotetrameric rather than monomeric annexin A2 had the ability to bind microsomes in a pH-dependent and Ca^{2+}-independent manner. Annexins

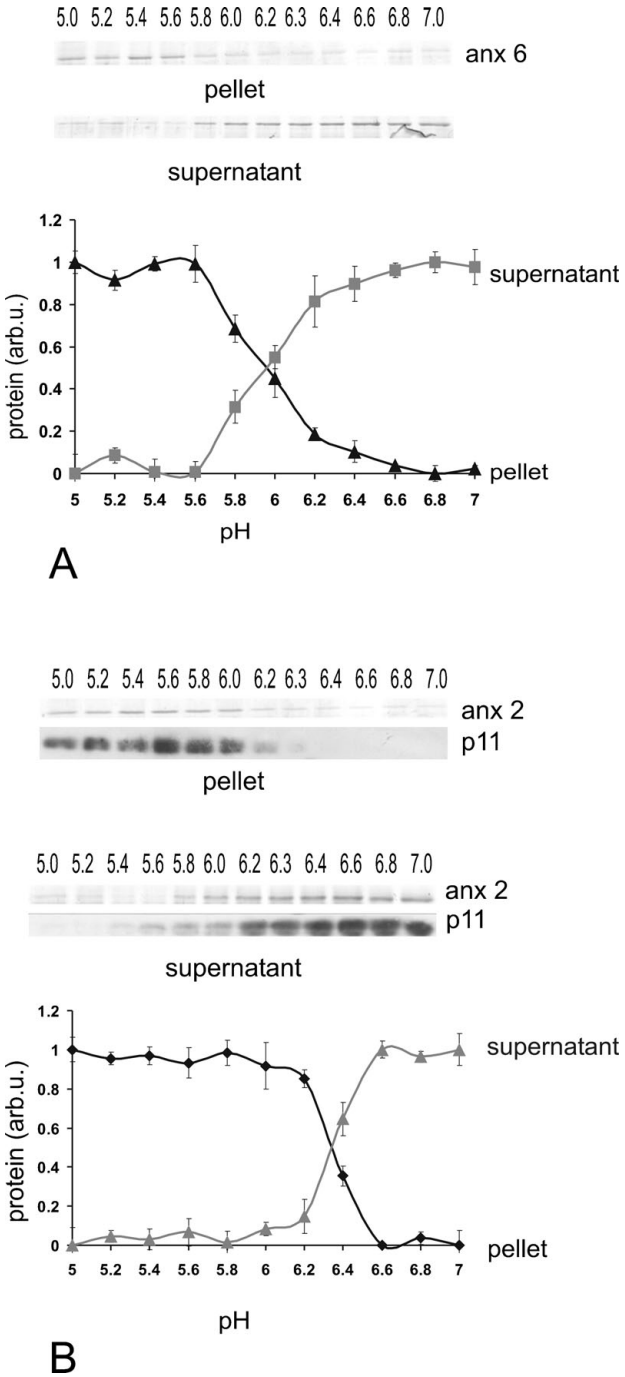

Figure 3 pH-dependent binding of annexins A6 and A2–S100A10 to microsomes

Porcine smooth muscle microsomes were prepared in the presence of 0.2 mM $CaCl_2$ to ensure membrane association of annexins. The microsomes were then pelleted by centrifugation and resuspended in buffer containing 100 mM Mes, 100 mM EGTA, 100 mM KCl at different pH values (from 5.0 to 7.0, in 0.2 pH unit increments) and incubated at 4 °C for 30 min. Membrane-bound annexins were then separated from the unbound proteins by centrifugation (20 min, 12000 ***g*** at 4 °C). The membrane-bound proteins in the pellet and the soluble proteins in the supernatant were analysed by SDS/PAGE followed by Coomassie staining for annexins A2 and A6, or Western blotting with monoclonal antibodies against S100A10 (p11). (**A**) Distribution of annexin A6 in fractions following the exposure to pH values ranging from 5.0 to 7.0. Results from one representative experiment are shown. The amounts (arbitrary units) of A6 in pellets and supernatants were plotted as a function of pH in the solution. Results are means ± S.D. for three experiments. (**B**) Distribution of annexin A2 and S100A10 between membrane-bound (pellet) and soluble (supernatant) fractions at different pH. Results are means ± S.D. for three experiments.

A1, A4 and A5 did not show a pH-dependent interaction with microsomes within the tested ranges of pH (results not shown).

Proteins tend to precipitate when the pH of the solution is near their isoelectric point, therefore we performed precipitation controls for annexin A2–S100A10 and annexin A6 (see the Experimental section). We found that annexins A2 and A6 started to aggregate at pH 4.5, which is well below the pH values at which their binding to the membranes was observed (see above).

We have previously shown that annexins A2 and annexin A6 preferentially interact with cholesterol-rich domains at the plasma membrane upon the increase of $[Ca^{2+}]_i$ [8]. In contrast with these observations, the Ca^{2+}-independent association of annexin A2–S100A10 and annexin A6 with microsomes was not significantly influenced by an alteration of the lipid composition by extraction of the microsomes with 2 % methyl-β-cyclodextrine to sequester cholesterol (not shown).

The A2–S100A10 heterotetramer is essential for pH-dependent membrane association

HEK 293 cells endogenously express both annexin A2 and S100A10, so it is possible that at least some of the recombinant annexin A2 formed a complex with the up-regulated endogenous S100A10. In order to investigate the Ca^{2+} sensitivities of A2 monomer and heterotetramer separately, we transfected hepatoblastoma HepG2 cells with recombinant fluorescent protein-tagged annexin A2 either alone, or together with S100A10 (Figure 4). These cells express virtually no endogenous annexin A2 or S100A10 [29]. In the absence of S100A10, annexin A2-CFP did not translocate to the plasma membrane of the butyrate-treated HepG2 cells following the decrease of pH_o to 6.8 (Figure 4A). When S100A10-YFP was co-expressed with annexin A2-YFP and annexin A6-CFP, a translocation of annexin A2-YFP to the plasma membrane was observed, similar to annexin A6 (Figure 4B). The monomeric annexin A2-YFP was largely pH-insensitive, showing a weak translocation at a pH_i of 5.4 (Figure 4C), whereas the annexin A2–S100A10-YFP complex showed the pH sensitivity, which was higher than that of annexin A6-CFP, translocating at a pH_i of 6.4 (Figure 4D). The observed binding was mediated by the annexin A2 part of the heterotetramer, rather than by S100A10, because S100A10-YFP expressed alone in HepG2 cells did not show a pH-sensitivity in response to any of the treatments (results not shown).

Distinct pH_i values are required for membrane-binding of individual annexins

To compare our biochemical with the microscopical results, we calibrated the pH_i using carboxy SNARF-4F in combination with 20 μM BAPTA. The cells expressing GFP-tagged annexins, and loaded with SNARF and BAPTA, were exposed to high K^+ buffers containing 20 μM nigericin at different pH values (ranging from 7.0 to 5.0, at 0.2 pH unit increments), which resulted in a controlled gradual acidification of the cytoplasm. Images of SNARF fluorescence and GFP localization were taken simultaneously and emission ratios of SNARF-4F were converted into pH values. A representative experiment for annexin A6-GFP is shown in the supplemental Figure 1 (http://www.BiochemJ.org/bj/409/bj4090065add.htm).

Similar experiments were performed with GFP-tagged annexins A1, A4, A5, A2–S100A10 and A2 core, a truncated form of annexin A2 lacking the amino-terminus and incapable of binding S100A10, and the results are summarized in Figure 5. In agreement with our biochemical results, recombinant annexin A6 and heterotetrameric annexin A2 were the most pH sensitive, showing a robust plasma membrane interaction at pH_i values of 6.2 and 6.6 respectively. The higher pH sensitivity of annexin A2–S100A10-GFP compared to annexin A6-GFP could also be observed in the nigericin-treated cells expressing annexin A2-CFP, S100A10-CFP and annexin A6-YFP (Video 6, http://www.BiochemJ.org/bj/409/bj4090065add.htm).

Dimerization of the annexin A2 core is essential for its pH-sensitivity

Using an annexin A2core-GFP fusion protein [30], we found the pH_i necessary for its association with the plasma membranes to be significantly lower (pH 5.4) than that of the heterotetrameric annexin A2–S100A10 (pH 6.6) (Figure 5). The heterotetramer has a total of eight annexin repeats, identical to the annexin A6 molecule. In order to assess the possible co-operative effects of core sequence multimerization, we created fusion constructs linking either two (A2c2c-YFP) or three (A2c2c2c-YFP) annexin A2 core sequences in-frame with YFP. Transient transfection of both plasmids in HEK 293 cells yielded proteins of expected molecular weights (96 and 130 kDa respectively, Figure 6A). A2c2c-YFP displayed a similar intracellular distribution as A2core-YFP, and A2c2c2c-YFP was largely excluded from the nucleus, probably due to its molecular mass (Figure 6B). In contrast with the annexin A2 core, both fusion proteins had the ability to bind the plasma membrane upon intracellular acidification, and both were more pH-sensitive than annexin A6 (Figure 6B). The annexin A2 core dimer construct interacted with the plasma membrane at a pH very similar to the annexin A2–S100A10 heterotetramer (Figure 6C), whereas the addition of a third core sequence in the annexin A2 core-trimer rendered the protein slightly more pH sensitive than the core dimer or the annexin A2–S100A10 complex, indicating that the binding was co-operative.

To investigate whether multimerization of annexin repeats was the reason for the proteins' pH sensitivity, we fused two sequences of annexin A4. Wild-type annexin A4 did not show a pH-induced membrane binding *in vitro* or as a fluorescent protein fusion in live cells (see above). Two repeats of the annexin A4 sequence were cloned in-frame with CFP, and the resulting protein annexin A4a4-CFP showed the expected size of approx. 90 kDa (supplemental Figure 2A, http://www.BiochemJ.org/bj/409/bj4090065add.htm). However, no acidification-induced membrane translocation was detected or any significant increase in pH sensitivity observed, compared to monomeric annexin A4-CFP (supplemental Figure 2B and 2C).

Recombinant fluorescent protein-tagged annexins A6 and A2–S100A10 translocate to the plasma membrane during hypoxia

HEK 293 cells were co-transfected with annexin A2-CFP and annexin A6-YFP, and placed under hypoxic conditions (1.5 % O_2) for 24 h, then fixed with ethanol inside the hypoxia workstation and examined on the confocal microscope. Under normoxic (20 % O_2) conditions, both proteins showed a diffuse cytoplasmic localization, without noticeable association with the plasma membrane (Figure 7A, graph). After 24 h of hypoxia, a significant proportion of annexin A6-YFP became associated with the plasma membrane, in contrast with monomeric annexin A2-CFP, which retained its cytoplasmic distribution (Figure 7B, graph). Adaptation to hypoxia is regulated by hypoxia inducible factor HIF-1, and the expression of its oxygen-regulated subunit HIF-1α was observed after 1 h in the chamber and persisted until 24 h afterwards (Figure 7C). We then performed similar experiments with the cells placed into a sealed chamber assembled at the microscope stage and exposed to the continuous flow of air and N_2 (1.5 % O_2, 98.5 % N_2). The cells were co-transfected with

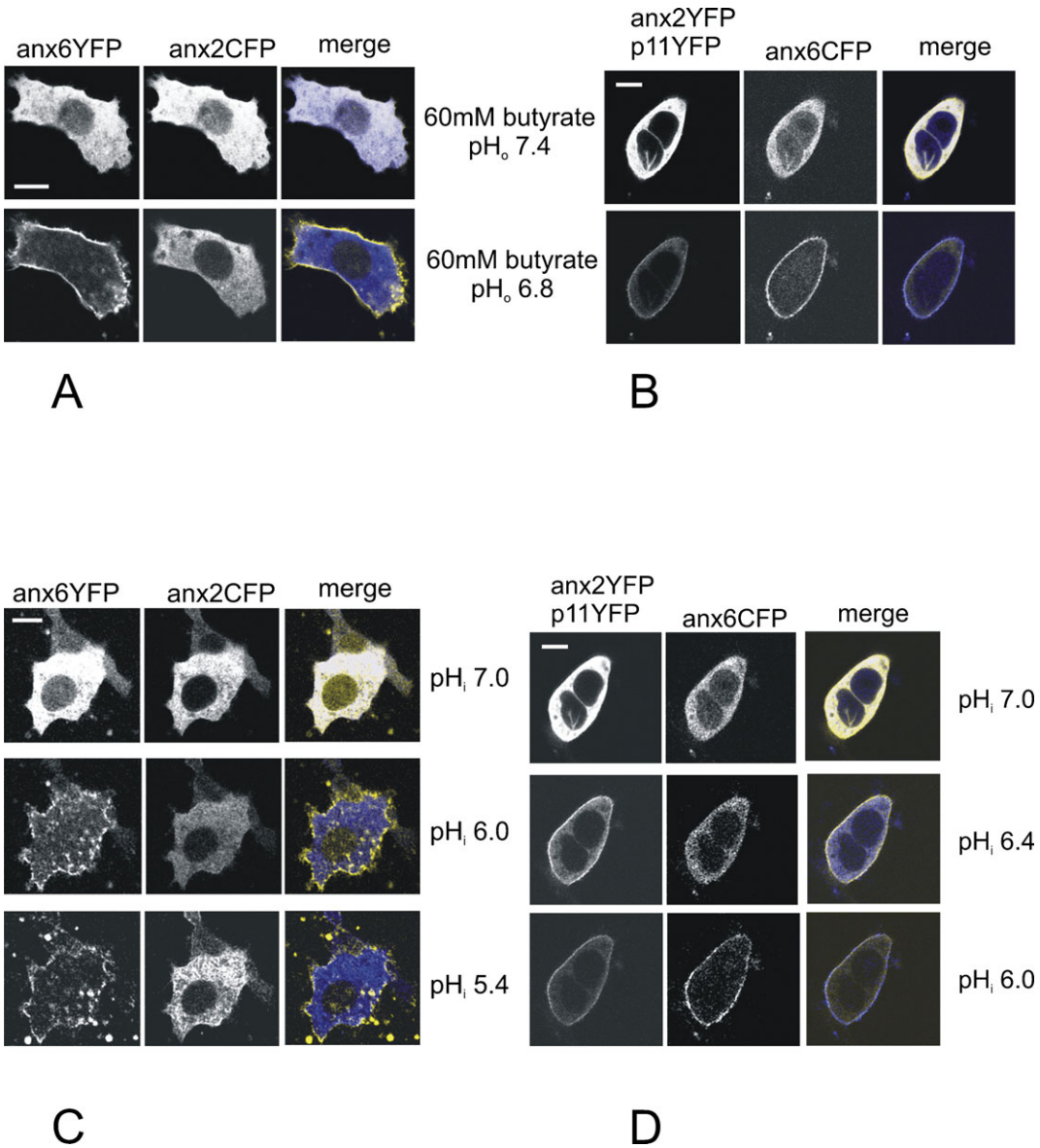

Figure 4 Heterotetramer formation is necessary for pH-dependent annexin A2 membrane association

HepG2 cells were co-transfected with plasmids expressing annexin A6-YFP and annexin A2-CFP (**A** and **C**) or annexin A6-CFP, annexin A2-YFP and S100A10-YFP (**B** and **D**). All cells were pre-loaded with 20 μM BAPTA/AM for 30 min before conducting experiments. (**A** and **B**) Cells were perfused with a buffer containing 60 mM Na^+-butyrate, pH$_o$ 7.4 (upper panel), or pH$_o$ 6.8 (lower panel). Annexin A6 is diffusely distributed in the cytoplasm (upper panel) and translocates to the plasma membrane upon reduction of the pH$_o$ to 6.8 (lower panel). Under the same conditions, the annexin A2 monomer remains cytoplasmic (**A**, lower panel), but translocates to the plasma membrane when co-expressed with S100A10 (p11) (**B**, lower panel). (**C** and **D**) Transfected HepG2 cells were incubated for 10 min in high K^+ buffer with indicated pH$_o$ values in the presence of 20 μM nigericin to allow equilibration of pH$_i$ with pH$_o$. Images were taken at each pH point. (**C**) Translocation of annexin A6-YFP to the plasma membrane at a pH$_o$ equivalent to a pH$_i$ of 6.0 (middle panel) and very weak translocation of the annexin A2 monomer at a pH$_i$ of 5.4 (lower panel). (**D**) Same field as shown in (**B**), after treatment with butyrate, cells were allowed to recover at a pH$_o$ of 7.4 and subjected to nigericin-induced acidification. Translocation of annexin A2–S100A10 occurs before translocation of annexin A6 at a pH$_i$ of 6.4 (middle panel). Annexin A6 translocates at pH 6.0 (lower panel). Bars = 10 μm, $n = 3$.

plasmids expressing annexin A2-YFP and S100A10-YFP, loaded with carboxy SNARF-4F/AM, and simultaneous evaluation of pH$_i$ and localization of the annexin A2 heterotetramer were performed by taking 5 frames at 3 seconds intervals after 15, 30, 45 and 60 min of hypoxia. pH$_i$ decreased from 7.2 to approx. 6.6 during the first hour of hypoxia (Figure 7D), however, the pH$_o$ of the HEPES-buffered Na^+-Tyrode's remained at 7.4, indicating that the decline of the pH$_i$ was induced by deficiencies of oxidative phosphorylation, or other hypoxia-related mechanisms. Translocation of annexin A2–S100A10 to the plasma membrane was observed after 30 min under hypoxic conditions, and remained stable after 1 h in the chamber (Figure 7E).

Endogenous annexins in smooth muscle tissue and HEK 293 cells associate with plasma membranes at low pH

Apart from the biochemical results on pH-dependent annexin-binding to smooth muscle microsomes, the results presented above were obtained using recombinant annexins tagged with fluorescent proteins and transiently expressed in cell lines. However, in order to demonstrate that the observed effects were not merely a function of chimeric proteins, we investigated the localization of endogenous annexins in human bladder smooth muscle bundles and in non-transfected HEK 293 cells exposed to a pH$_o$ of 5.0 (supplemental Figure 3A, http://www.BiochemJ.

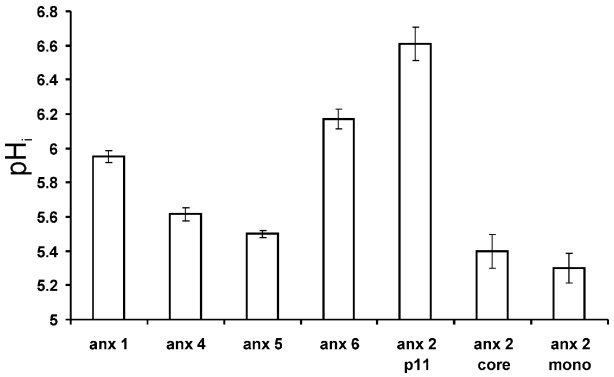

Figure 5 Annexins show distinct pH sensitivities of membrane association

HEK 293 cells were co-transfected with annexins tagged with GFP, loaded with 20 μM BAPTA/AM and 6 μM carboxy SNARF-4F/AM and the pH$_i$ was lowered by 20 μM nigericin and a graded decrease of pH$_o$. The decrease in pH$_i$ was monitored simultaneously with the annexin localization. The graphs shows the values of pH$_i$ necessary for the translocation of GFP-tagged annexins A1, A4, A5, A6, A2–S100A10 and A2-core to the plasma membrane of HEK 293 cells and of monomeric annexin A2-GFP in HepG2 cells. The results represent an average of three independent experiments (n = 5 cells per experiment).

org/bj/409/bj4090065add.htm). Exposure of the muscle, 'relaxed' in a Ca^{2+}-free solution, to a pH$_o$ of 5.0 in the presence of 100 μM BAPTA/AM to ensure the removal of intracellular Ca^{2+}, prompted the relocation of annexin A6 to the plasmalemma. In cultured HEK 293 cells at a low pH$_o$, endogenous annexins redistributed analogous to the recombinant ones: at a pH$_o$ of 5.0, annexins A6 and A2 became associated with the plasma membrane and annexin A5 retained its diffuse cytoplasmic localization.

The biochemical characterization of pH-dependent membrane-association of endogenous annexins from HEK293 cells (supplemental Figure 3B) with membranes closely resemble the results obtained for porcine smooth muscle microsomes, demonstrating that both recombinant and endogenous annexins react alike when subjected to low pH values.

DISCUSSION

A large number of proteins have the ability to interact with cellular membranes in a pH-dependent manner, thus acting as pH sensors: cytochrome *c*, Bcl-xL and chromogranins A and B have been shown to bind lipid vesicles at acidic pH values [31–33], also, ARF1 is associated with endosomal membranes in a pH-dependent manner [34].

The annexins comprise over 50 members, are found in all eukaryotic phyla [35], and exhibit a similar molecular structure. Their characteristic ability to bind negatively charged phospholipids in a Ca^{2+}-dependent manner has been extensively investigated [7,36]. Here, we focus on their ability to interact with membranes in a Ca^{2+}-independent fashion. We constructed fusions of annexins A1, A2, A4, A5 and A6 with fluorescent proteins, and monitored their localization in response to changes in pH$_i$ caused by treatment with the pH$_i$-reducing agent sodium butyrate, extracellular acidification and hypoxia. Our study compares for the first time the Ca^{2+}-independent membrane-binding activities of different annexins in live cells, and demonstrates that in the higher, more physiological pH range (6.2 and 6.6) only annexin A6 and annexin A2–S100A10 are capable of Ca^{2+}-independent membrane association. These results are supported by the results obtained for endogenous annexins from human bladder smooth muscle tissue, HEK 293 cells and porcine

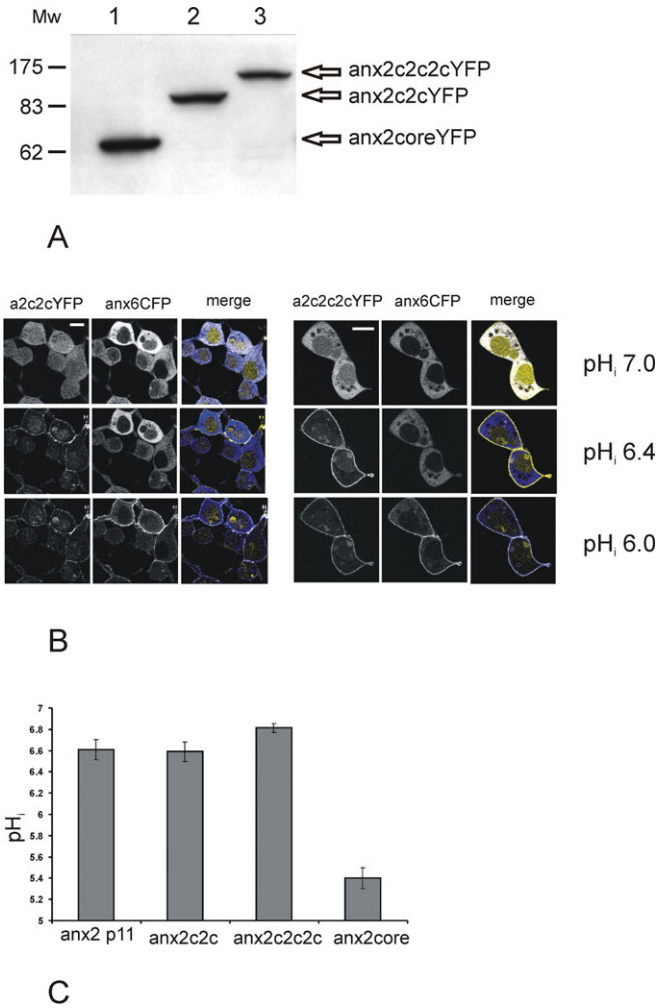

Figure 6 The fusion of two or three annexin A2 core sequences increases the pH sensitivity of the resulting proteins

(**A**) Annexin A3 core-YFP, A2c2c-YFP or annexin A2c2c2c-YFP, containing one, two or three copies of the A2 core sequence, as well as the annexin A2 core, were expressed in HEK 293 cells. At 48 h post-transfection, the cells were collected, lysed and analysed by SDS/PAGE followed by Western blotting with a monoclonal antibody against GFP. Lane 1, annexin A2 core-YFP; lane 2, annexin A2c2c-YFP; and lane 3, annexin A2c2c2c-YFP. Mw, molecular mass markers (kDa). (**B**) HEK 293 cells co-expressing annexin A6-CFP with either Anx2c2c (left) or Anx2c2c2c (right) were incubated in high K^+ buffer with 20 μM nigericin and subjected to the nigericin-mediated decrease of pH$_i$. At a pH$_i$ of 7.0, all proteins are localized in the cytoplasm (upper panels). A decrease in pH$_i$ to 6.4 induces a translocation of annexin A2c2c and A2c2c2c to the plasma membrane (middle panels), and a pH$_i$ of 6.0 causes annexin A6-CFP to interact with the plasma membrane (lower panels). (**C**) Cells expressing annexin A2c2c-YFP or A2c2c2c-YFP were loaded with 20 μM BAPTA/AM and 6 μM carboxy SNARF-4F/AM and subjected to nigericin-induced gradual intracellular acidification. The graph shows the pH$_i$ values necessary for membrane association of both constructs, with the values for annexin A2 core and the annexin A2–S100A10 (p11) complex shown for comparison. The results are the means of three experiments (n = 5 cells per experiment). Bars = 10 μm.

microsomes, showing a pH-dependent membrane association of heterotetrameric annexin A2 and annexin A6.

The acidification-mediated membrane binding of annexins A6 and A2–S100A10 is Ca^{2+}-independent and not influenced by membrane lipid composition

Extracellular acidification reduces the activity of Na^+/H^+ exchangers and other regulators of pH homoeostasis, and as a consequence decreases pH$_i$ [24]. When the pH$_i$ was monitored in HEK 293 cells exposed to a pH$_o$ of 3.0, we found that it was

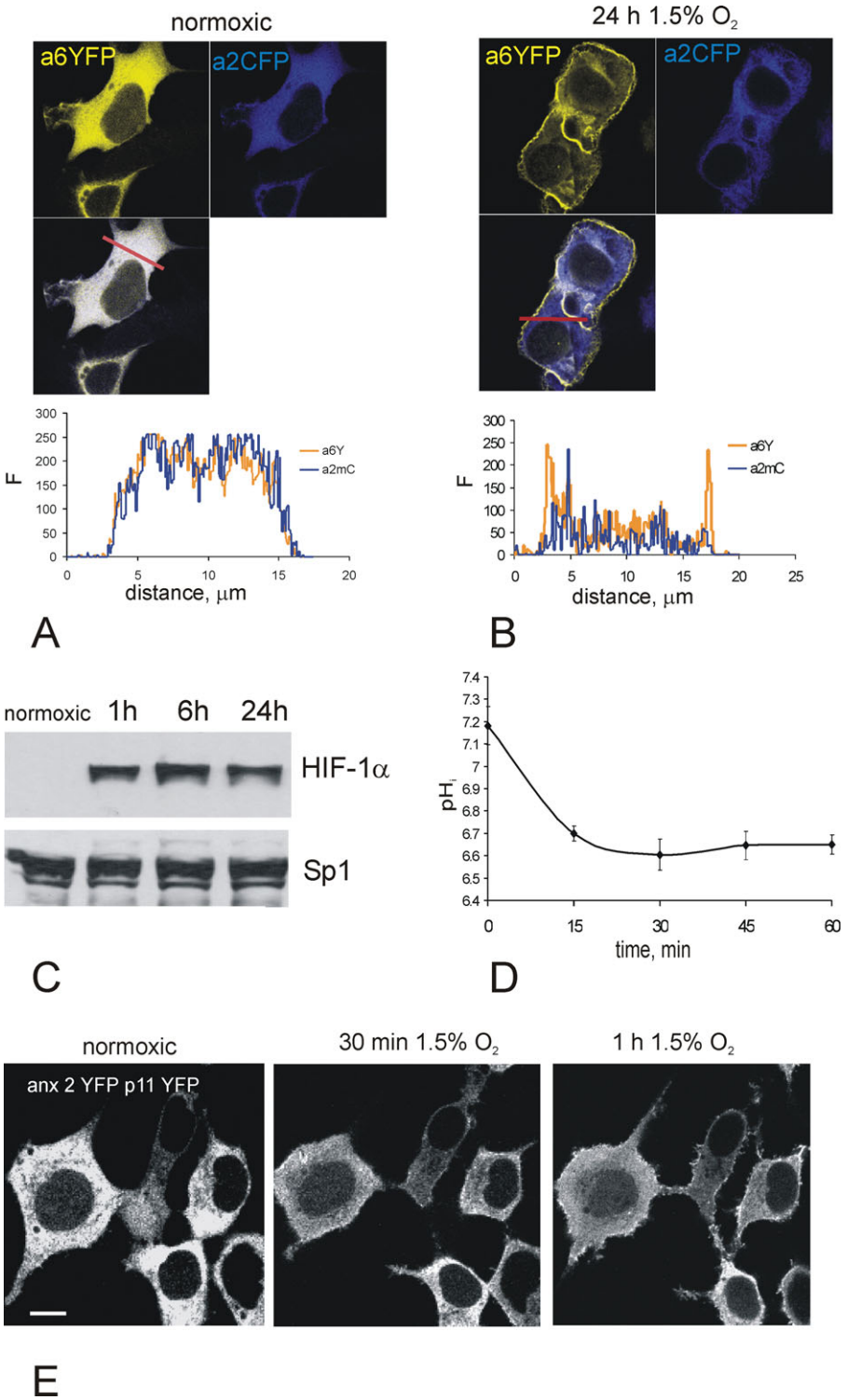

Figure 7 Hypoxia induces the membrane association of annexin A6 and the annexin A2–S100A10 heterotetramer

HEK 293 cells co-transfected with annexin A6-YFP and monomeric annexin A2-CFP were either grown under normoxic conditions (**A**) or placed into a hypoxic chamber (1.5 % O_2) for 24 h (**B**). The cells were fixed with cold ethanol, re-hydrated with PBS, mounted and examined in a Zeiss LSM 510 META; 30 cells chosen at random were examined in each preparation and representative images selected for presentation. To determine the extent of membrane association of each protein, an arbitrary axis was chosen (shown in red in the merged image), and intensity profiles of the blue and yellow signals were calculated (graphs). (**C**) HEK 293 cells were placed under hypoxic conditions for the periods of 1, 6 or 24 h, and nuclear-enriched lysates prepared. An aliquot of nuclear-enriched protein (50 μg) was separated by SDS/PAGE (7.5 % gel) and analysed by Western blotting with a polyclonal antibody against HIF-1α. The membranes were re-incubated with a polyclonal antibody against nuclear marker Sp1 to control for equal loading. (**D**) Cells were grown on coverslips, loaded with carboxy SNARF-4F/AM and exposed to hypoxic conditions (1.5 % O_2) while examining them under the confocal microscope. A series of 5 images was taken every 15 min for 60 min, and SNARF emission ratios were converted to pH_i values (calibration described in the Experimental section). A mean of 2 experiments is shown on the graph. (**E**) Cells co-expressing annexin A2-YFP and S100A10-YFP were grown on coverslips, placed into an air-tight chamber mounted on the stage of a Zeiss LSM confocal microscope in Ca^{2+}-free Na^+-Tyrode's buffer, pH 7.4, and hypoxia (1.5 % O_2) was induced by pumping a mixture of air and N_2. Images were taken every 15 min for 60 min. Bar = 10 μm, data from a representative experiment are shown ($n = 3$).

gradually declining to approx. 6.1. When the pH_i dropped below 6.3, annexin A6-YFP, but not annexin A2-CFP, became associated with the plasma membrane, which allowed the assumption that the pH-induced binding was Ca^{2+}-independent. However, intracellular acidification often correlates with a marked increase in $[Ca^{2+}]_i$ due to the altered activity of calcium-entry channels, as well as release from the intracellular stores (ER and mitochondria) and decreased affinity for Ca^{2+}-binding proteins [26,28]. It was therefore necessary to exclude the observed translocations after acidification that were mediated by a rise in $[Ca^{2+}]_i$. We found that a decrease of pH_i caused a concomitant increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, therefore the cells were loaded with the Ca^{2+} chelator BAPTA/AM. Loading of cells with 20 μM BAPTA/AM had no effect on the butyrate-induced changes of pH_i. Although both the Fluo-3 fluorescence and the BAPTA affinity for Ca^{2+} are pH-sensitive, in the BAPTA-loaded cells, but not in the BAPTA-free cells, the concomitant increase of Fluo-3 fluorescence was abolished, indicating that Ca^{2+} was indeed buffered.

When the pH_i was reduced in cells treated with sodium butyrate, only annexin A6 and the heterotetrameric form of A2–S100A10 associated with the plasma membrane. *In vitro,* in the presence of 100 mM EGTA, both annexin A2 and S100A10 bound to microsomes (half-maximum binding at pH 6.4), with an identical distribution between the soluble and membrane-bound states, indicating that the heterotetrameric rather than monomeric form was responsible for pH-dependent, Ca^{2+}-independent association. Annexin A6 interacted with microsomal membranes in a Ca^{2+}-independent manner (half-maximal binding at pH 6.0), while annexin A1, A4, and A5 did not show a pH-dependent interaction with microsomes. Selective extraction of cholesterol had no effect on the binding efficiency of annexin A6 or annexin A2–S100A10. These results differ from previous results on Ca^{2+}-mediated membrane binding at neutral pH, which is strongly affected by the composition of the lipid bilayer [18,19]. They also indicate that the less discriminative hydrophobic interactions might bring about the pH-mediated membrane association, which is in agreement with the biophysical models [37].

To correlate the biochemical assays with the situation *in vivo*, we monitored the localization of GFP-tagged annexin A1, A2, A4, A5, A6, A2-core and A2–S100A10 in nigericin-treated cells in high K^+ buffers with different pH_o. Analogous to the gradient of annexin calcium sensitivities [9], we have established a pH-gradient for annexin–membrane binding. The pH_i required for the translocation of annexin A2–S100A10 was 6.6, followed by annexin A6 (6.2) and annexin A1 (5.9). The pH_i of annexin A4 and A5, as well as the annexin A2-core and monomeric annexin A2 (measured in HepG2 cells) was around 5.3, which correlated well with the pH determined by the microsome-binding assays.

Dimerization of the annexin A2 core sequence is obligatory for a co-operative pH-dependent membrane association

We show that the dimerization of annexin A2 within the heterotetramer is essential for acidification-induced membrane binding of the complex, and that the pH sensitivity of the heterotetramer is greater than that of annexin A6. Annexin A2 but not S100A10 was the mediator of the pH-induced membrane binding of the heterotetramer, which gave rise to the idea that it was the number of annexin repeats which rendered the protein pH-sensitive. Both annexin A6 and the annexin A2-heterotetramer have eight repeats, therefore we designed an annexin which exclusively consisted of repeats by fusing two annexin A2 core sequences. We found that the mere dimerization of the annexin A2 core rendered the protein pH-sensitive, and that the pH_i necessary for its membrane binding was virtually identical to that of the annexin A2–S100A10 heterotetramer. Interestingly, the annexin A2c2c protein (A2 core dimer) was also significantly more Ca^{2+} sensitive than the annexin A2 core when binding to the membranes at neutral pH (K. Monastyrskaya, unpublished work). The addition of a third core sequence produced a protein (A2c2c2c) with slightly higher pH sensitivity than that of the dimeric core, indicating that the pH-dependent membrane interaction of these amino acid sequences was co-operative. The amino acid composition of the cores was important for binding: a similar dimerization of annexin A4 did not result in a pH-sensitive protein. Our results are in accordance with the predictions by Golczak et al. [38] who calculated a pH-sensitive behaviour of annexin A2 based on its amino acid sequence.

Hypoxia causes intracellular acidification and promotes the interaction of annexin A6-YFP and annexin A2–S100A10-YFP with the plasma membrane

Exposure of annexin A6-YFP-expressing cells to hypoxic conditions for 24 h resulted in the association of annexin A6 with the plasma membrane. Hypoxia and ischaemia are associated with a decrease in pH_i: a mean pH_i of 5.3 was demonstrated in hippocampal neurons [39,40]. Subjecting the cells to hypoxia in our experimental setup induced a robust expression of the oxygen-regulated HIF-1 subunit HIF-1α, observed within 1 h of exposure and with a concomitant drop in pH_i. Within the first 30 min, the pH_i in the hypoxic cells decreased to approx. 6.6, however no translocation of annexin A6 could be observed during the first hour, in contrast with the more prolonged treatment (24 h). It was not possible to monitor the pH_i in the cells placed in the hypoxia workstation, but pH_o of the bicarbonate-buffered cultivation medium was decreased during this time (from 7.4 to 6.9). This might have promoted a further decrease of pH_i and thus assisted the membrane association of annexin A6. When annexin A2 was co-expressed with S100A10, the resulting heterotetramer gained the ability to translocate to the plasma membrane after 30 min under hypoxic conditions. The pH_i values required for the membrane association of the recombinant annexin A2–S100A10 in normal and hypoxic conditions correlate very well, which indicates that it is the hypoxia-induced intracellular acidification and not the reduction of O_2 *per se* which drives the interaction.

This is the first report of a pH-induced membrane association of the recombinant annexins *in vivo* under hypoxic conditions. Although most annexins have the ability to interact with membranes at low pH values, the sensitivity of this interaction varies greatly depending upon the amino acid sequence of the individual proteins, where annexin A2-heterotetramer and annexin A6 are the most pH-sensitive. Annexins A6 and annexin A2–S100A10 have been reported to possess a channel-modulating activity [41–43], the process for which their association with membranes is a necessary first step. Interestingly, short-chain fatty acids including butyrate are known to induce apoptosis in colon cancer cells *in vitro* and *in vivo*, however their mode of action is poorly defined [44]. Our observations, carried out in living cells, add an important facet to the mechanisms of the membrane interaction of annexins and open a new investigative avenue for potential functions of these proteins under the low pH conditions occurring in ischaemia and cancer.

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REFERENCES

1 Schmidt, W. K. and Moore, H. P. (1995) Ionic milieu controls the compartment-specific activation of pro-opiomelanocortin processing in AtT-20 cells. Mol. Biol. Cell. **6**, 1271–1285
2 Lipton, P. (1999) Ischemic cell death in brain neurons. Physiol. Rev. **79**, 1431–1568
3 Shrode, L. D., Tapper, H. and Grinstein, S. (1997) Role of intracellular pH in proliferation, transformation and apoptosis. J. Bioenerg. Biomembr. **29**, 393–399
4 Fitts, R. H. (1994) Cellular mechanisms of muscle fatigue. Physiol. Rev. **74**, 49–94
5 Ivanov, S., Liao, S. Y., Ivanova, A., Danilkovitch-Miagkova, A., Tarasova, N., Weirich, G., Merrill, M. J., Proescholdt, M. A., Oldfield, E. H. et al. (2001) Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am. J. Pathol. **158**, 905–919
6 Pouyssegur, J., Dayan, F. and Mazure, N. M. (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature **441**, 437–443
7 Gerke, V. and Moss, S. E. (2002) Annexins: from structure to function. Physiol. Rev. **82**, 331–371
8 Draeger, A., Wray, S. and Babiychuk, E. B. (2005) Domain architecture of the smooth-muscle plasma membrane: regulation by annexins. Biochem. J. **387**, 309–314
9 Monastyrskaya, K., Babiychuk, E. B., Hostettler, A., Rescher, U. and Draeger, A. (2007) Annexins as intracellular calcium sensors. Cell Calcium **41**, 207–219
10 Golczak, M., Kicinska, A., Bandorowicz-Pikula, J., Buchet, R., Szewczyk, A. and Pikula, S. (2001) Acidic pH-induced folding of annexin VI is a prerequisite for its insertion into lipid bilayers and formation of ion channels by the protein molecules. FASEB J. **15**, 1083–1085
11 Kim, Y. E., Isas, J. M., Haigler, H. T. and Langen, R. (2005) A helical hairpin region of soluble annexin B12 refolds and forms a continuous transmembrane helix at mildly acidic pH. J. Biol. Chem. **280**, 32398–32404
12 Kohler, G., Hering, U., Zschornig, O. and Arnold, K. (1997) Annexin V interaction with phosphatidylserine-containing vesicles at low and neutral pH. Biochemistry **36**, 8189–8194
13 Isas, J. M., Cartailler, J. P., Sokolov, Y., Patel, D. R., Langen, R., Luecke, H., Hall, J. E. and Haigler, H. T. (2000) Annexins V and XII insert into bilayers at mildly acidic pH and form ion channels. Biochemistry **39**, 3015–3022
14 Hegde, B. G., Isas, J. M., Zampighi, G., Haigler, H. T. and Langen, R. (2006) A novel calcium-independent peripheral membrane-bound form of annexin B12. Biochemistry **45**, 934–942
15 Golczak, M., Kirilenko, A., Bandorowicz-Pikula, J. and Pikula, S. (2001) Conformational states of annexin VI in solution induced by acidic pH. FEBS Lett. **496**, 49–54
16 Grewal, T., Heeren, J., Mewawala, D., Schnitgerhans, T., Wendt, D., Salomon, G., Enrich, C., Beisiegel, U. and 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
17 Hoekstra, D., Buist-Arkema, R., Klappe, K. and Reutelingsperger, C. P. (1993) Interaction of annexins with membranes: the N-terminus as a governing parameter as revealed with a chimeric annexin. Biochemistry **32**, 14194–14202
18 Babiychuk, E. B. and Draeger, A. (2000) Annexins in cell membrane dynamics: Ca^{2+}-regulated association of lipid microdomains. J. Cell Biol. **150**, 1113–1123
19 Babiychuk, E. B., Palstra, R. J., Schaller, J., Kampfer, U. and Draeger, A. (1999) Annexin VI participates in the formation of a reversible, membrane-cytoskeleton complex in smooth muscle cells. J. Biol. Chem. **274**, 35191–35195
20 Monastyrskaya, K, Babiychuk, E. B., Schittny, J. C., Rescher, U., Gerke, V., Mannherz, H. G. and Draeger, A. (2003) The expression levels of three raft-associated molecules in cultivated vascular cells are dependent on culture conditions. Cell. Mol. Life Sci. **60**, 2702–2709
21 Thomas, J. A., Buchsbaum, R. N., Zimniak, A. and Racker, E. (1979) Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated *in situ*. Biochemistry **18**, 2210–2218
22 Babiychuk, E. B. and Draeger, A. (2006) Biochemical characterization of detergent-resistant membranes: a systematic approach. Biochem. J. **397**, 407–416
23 Stroka, D. M., Burkhardt, T., Desbaillets, I., Wenger, R. H., Neil, D. A., Bauer, C., Gassmann, M. and Candinas, D. (2001) HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. FASEB J. **15**, 2445–2453
24 Saito, Y., Ozawa, T. and Nishiyama, A. (1990) Effects of intra- and extracellular H^+ and Na^+ concentrations on Na(+)-H^+ antiport activity in the lacrimal gland acinar cells. Pflugers Arch. **417**, 382–390
25 Yawata, K., Nagata, M., Narita, A. and Kawai, Y. (2001) Effects of long-term acidification of extracellular pH on ATP-induced calcium mobilization in rabbit lens epithelial cells. Jpn. J. Physiol. **51**, 81–87
26 Wakabayashi, I., Poteser, M. and Groschner, K. (2006) Intracellular pH as a determinant of vascular smooth muscle function. J. Vasc. Res. **43**, 238–250
27 Duquette, R. A. and Wray, S. (2001) pH regulation and buffering power in gastric smooth muscle. Pflugers Arch. **442**, 459–466
28 Austin, C. and Wray, S. (2000) Interactions between Ca(2+) and H(+) and functional consequences in vascular smooth muscle. Circ. Res. **86**, 355–363
29 Puisieux, A., Ji, J. and Ozturk, M. (1996) Annexin II up-regulates cellular levels of p11 protein by a post-translational mechanisms. Biochem. J. **313**, 51–55
30 Rescher, U., Zobiack, N. and Gerke, V. (2000) Intact Ca^{2+}-binding sites are required for targeting of annexin 1 to endosomal membranes in living HeLa cells. J. Cell Sci. **113**, 3931–3938
31 Rytomaa, M., Mustonen, P. and Kinnunen, P. K. (1992) Reversible, nonionic and pH-dependent association of cytochrome *c* with cardiolipin-phosphatidylcholine liposomes. J. Biol. Chem. **267**, 22243–22248
32 Thuduppathy, G. R., Terrones, O., Craig, J. W., Basanez, G. and Hill, R. B. (2006) The N-terminal domain of Bcl-xL reversibly binds membranes in a pH-dependent manner. Biochemistry **45**, 14533–14542
33 Yoo, S. H. (1995) Purification and pH-dependent secretory vesicle membrane binding of chromogranin B. Biochemistry **34**, 8680–8686
34 Gu, F. and Gruenberg, J. (2000) ARF1 regulates pH-dependent COP functions in the early endocytic pathway. J. Biol. Chem. **275**, 8154–8160
35 Morgan, R. O., Martin-Almedina, S., Iglesias, J. M., Gonzalez-Florez, M. I. and Fernandez, M. P. (2004) Evolutionary perspective on annexin calcium-binding domains. Biochim. Biophys. Acta. **1742**, 133–140
36 Gerke, V., Creutz, C. E. and Moss, S. E. (2005) Annexins: linking Ca^{2+} signalling to membrane dynamics. Nat. Rev. Mol. Cell Biol. **6**, 449–461
37 Hegde, B. G., Isas, J. M., Zampighi, G., Haigler, H. T. and Langen, R. (2006) A novel calcium-independent peripheral membrane-bound form of annexin B12. Biochemistry **45**, 934–942
38 Golczak, M., Kirilenko, A., Bandorowicz-Pikula, J. and Pikula, S. (2001) N- and C-terminal halves of human annexin VI differ in ability to form low pH-induced ion channels. Biochem. Biophys. Res. Commun. **284**, 785–791
39 Sheldon, C. and Church, J. (2002) Intracellular pH response to anoxia in acutely dissociated adult rat hippocampal CA1 neurons. J. Neurophysiol. **87**, 2209–2224
40 Chesler, M. (2005) Failure and function of intracellular pH regulation in acute hypoxic-ischemic injury of astrocytes. Glia **50**, 398–406
41 Sobota, A., Cusinato, F. and Luciani, S. (1990) Identification and purification of calpactins from cardiac muscle and their effect on Na^+/Ca^{2+} exchange activity. Biochem. Biophys. Res. Commun. **172**, 1067–1072
42 Naciff, J. M., Behbehani, M. M., Kaetzel, M. A. and Dedman, J. R. (1996) Annexin VI modulates Ca^{2+} and K^+ conductances of spinal cord and dorsal root ganglion neurons. Am. J. Physiol. **71**, C2004–C2015
43 Riquelme, G., Llanos, P., Tischner, E., Neil, J. and Campos, B. (2004) Annexin 6 modulates the maxi-chloride channel of the apical membrane of syncytiotrophoblast isolated from human placenta. J. Biol. Chem. **279**, 50601–50608
44 Matthews, G. M., Howarth, G. S. and Butler, R. N. (2007) Short-chain fatty acid modulation of apoptosis in the Kato III human gastric carcinoma cell line. Cancer Biol. Ther. **6**, 1051–1057

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