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Brefeldin A influences the cell surface abundance and intracellular pools of low and high ouabain affinity Na^+, K^+-ATPase α subunit isoforms in articular chondrocytes

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Summary. The catalytic α isoforms of the Na^+, K^+-ATPase and stimuli controlling the plasma membrane abundance and intracellular distribution of the enzyme were studied in isolated bovine articular chondrocytes which have previously been shown to express low and high ouabain affinity α isoforms (α1 and α3 respectively; $\alpha 1 >> \alpha 3$). The Na^+, K^+-ATPase density of isolated chondrocyte preparations was quantified by specific ^{3}H-ouabain binding. Long-term elevation of extracellular medium [Na^+] resulted in a significant (31%; $p<0.05$) upregulation of Na^+, K^+-ATPase density and treatment with various pharmacological inhibitors (Brefeldin A, monensin and cycloheximide) significantly ($p<0.001$) blocked the upregulation. The subcellular distribution of the Na^+, K^+-ATPase α isoforms was examined by immunofluorescence confocal laser scanning microscopy which revealed predominantly plasma membrane immunostaining of α subunits in control chondrocytes. In Brefeldin A treated chondrocytes exposed to high [Na^+], Na^+, K^+-ATPase α isoforms accumulated in juxta-nuclear pools and plasma membrane Na^+, K^+-ATPase density monitored by ^{3}H-ouabain binding was significantly down-regulated due to Brefeldin A mediated disruption of vesicular transport. There was a marked increase in intracellular α1 and α3 staining suggesting that these isoforms are preferentially upregulated following long-term exposure to high extracellular [Na^+]. The results demonstrate that Na^+, K^+-ATPase density in chondrocytes is elevated in response to increased extracellular [Na^+] through *de novo* protein synthesis of new pumps containing α1 and α3 isoforms, delivery via the endoplasmic reticulum-Golgi complex constitutive secretory pathway and insertion into the plasma membrane.

Key words: Chondrocyte, cartilage, Na^+, K^+-ATPase, α isoforms, Upregulation, Brefeldin A, Cycloheximide, Monensin, Ouabain, Confocal microscopy

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Introduction

The Na^+, K^+-ATPase ((Na^+ + K^+)-stimulated adenosine triphosphatase; E.C. 3.6.1.37) is a Mg^{2+}-dependent pump responsible for maintaining the transmembrane gradients of Na^+ and K^+ (for reviews see Sweadner, 1989, 1995). It catalyses the active uptake of K^+ and extrusion of Na^+ at the expense of hydrolysing ATP to ADP and P_i. The uphill transport establishes steep concentration gradients for Na^+ and K^+ that are harnessed by other membrane transport proteins for a variety of essential physiological functions (Rossier et al., 1987). The Na^+, K^+-ATPase consists of a 112 kD catalytic α subunit, a 45-55 kD ß subunit (Fambrough et al., 1994; Sweadner, 1995) and a smaller 58 amino acid γ subunit with a molecular weight of 6.5 kD (Mercer et al., 1993). There are functional differences in the Na^+, K^+-ATPase from various tissues. These include differences in affinity for cardiac glycosides such as digitalis and ouabain (Juhaszova and Blaustein, 1997) and for Na^+ and K^+, attributable to the catalytic α isoforms (Sweadner, 1989) of which four different isoform variants have been described so far (Shamraj and Lingrel, 1994). Of the four known α isoform genes, α1 appears to be ubiquitously expressed in all cells and tissues examined to date (Sweadner, 1989, 1995; Lingrel et al., 1990; Shamraj and Lingrel, 1994). The α2 and α3 isoforms are expressed primarily in electrically excitable tissues; α2 has been found mainly in the brain, heart and skeletal muscle (Sweadner, 1995); α3 is most restricted in its tissue expression, being found primarily in neurons in the brain (Urayama et al., 1989; Sweadner, 1995) and in human and primate myocardium (Shamraj et al., 1993; Sweadner et al., 1994); α4 appears to be unique to the testis (Shamraj and Lingrel, 1994).

Chondrocytes exist in an extracellular environment where the concentration of free cations is high (350-450mM Na^+, 8-15mM K^+) (Lesperance et al., 1992). This is due to the fixed negative charges on the sulphate and carboxylate groups of glycosaminoglycans (predominantly chondroitin sulphate and keratan sulphate), constituents of extracellular matrix proteoglycans. Glycosaminoglycans control the ionic

composition (particularly [Na^+]) of the extracellular matrix of cartilage (Mobasheri, 1998). The extracellular [Na^+] is directly related to the concentration of the fixed negative charges, and hence to the local concentration of glycosaminoglycans. In addition, the local extracellular [Na^+] in the vicinity of chondrocytes will vary during static joint loading, as fluid expression from cartilage will increase the local glycosaminoglycan concentrations, simultaneously increasing the local extracellular [Na^+] (Maroudas, 1979). The intracellular [Na^+] will depend on chondrocyte permeability and extracellular [Na^+]; although the permeability of cations such as Na^+ and K^+ across the chondrocyte plasma membrane is low, they tend to move down their electrochemical gradient; thus K^+ tends to move out of and Na^+ into chondrocytes. All animal cells need to maintain a low Na^+:K^+ ratio vital for the optimal activity of many metabolic enzymes. They achieve this by expressing a specific Na^+/K^+ pump in the plasma membrane.

Isolated bovine chondrocytes have been shown to express multiple α and ß Na^+, K^+-ATPase isoforms; the expression of the α1 and α3 isoform have been demonstrated at the protein and mRNA levels (Mobasheri et al., 1997a,b, 1998). The plasma membrane density of the chondrocyte Na^+, K^+-ATPase is relatively high (~$1.5x10^5$ Na^+, K^+-ATPase pump sites/cell) for these small cells (8-12μm average diameter). The plasma membrane density of the Na^+, K^+-ATPase in chondrocytes is sensitive to the ionic and osmotic conditions of the extracellular environment (Mobasheri et al., 1997a,b). If extracellular [Na^+] is experimentally elevated, Na^+ ions gain entry to the cytosol in chondrocytes via ion channels and transporter proteins abundantly expressed in these cells and increase intracellular [Na^+]. These include tetrodotoxin sensitive Na^+ channels (Wright et al., 1992), amiloride sensitive Na^+ channels (Trujillo et al., 1999), the Na^+-H^+ antiporter and the $Na^+/K^+/2Cl^-$ cotransport carrier (Mobasheri, A., unpublished observations). Increased intracellular [Na^+] is a stimulus for Na^+, K^+-ATPase upregulation (Brodie and Sampson, 1989) and since Na^+, K^+-ATPase abundance is regulated by intracellular [Na^+], the expression and plasma membrane density of the Na^+, K^+-ATPase is likely to vary with changing extracellular [Na^+] and play an important role in the adaptation of the chondrocyte to changes in its extracellular environment. Here, the effect of various inhibitors on the expression and density of the Na^+, K^+-ATPase was studied in order to determine whether the observed upregulation is a result of *de novo* protein synthesis or recruitment from intracellular stores. The effects of pharmacological inhibitors of protein synthesis (cycloheximide), anterograde vesicular transport (Brefeldin A) and secretion from the Golgi complex, (monensin) on plasma membrane Na^+, K^+-ATPase abundance were studied in conjunction with long-term changes in the extracellular [Na^+] employed as a stimulus for upregulation (Fig. 1). A quantitative ^3H-ouabain binding approach was used to measure Na^+, K^+-ATPase abundance on the plasma membrane and determine its absolute density in the presence and absence of the drugs. Immunofluorescence confocal microscopy was used to examine the expression of the catalytic α isoforms of the Na^+, K^+-ATPase (employing

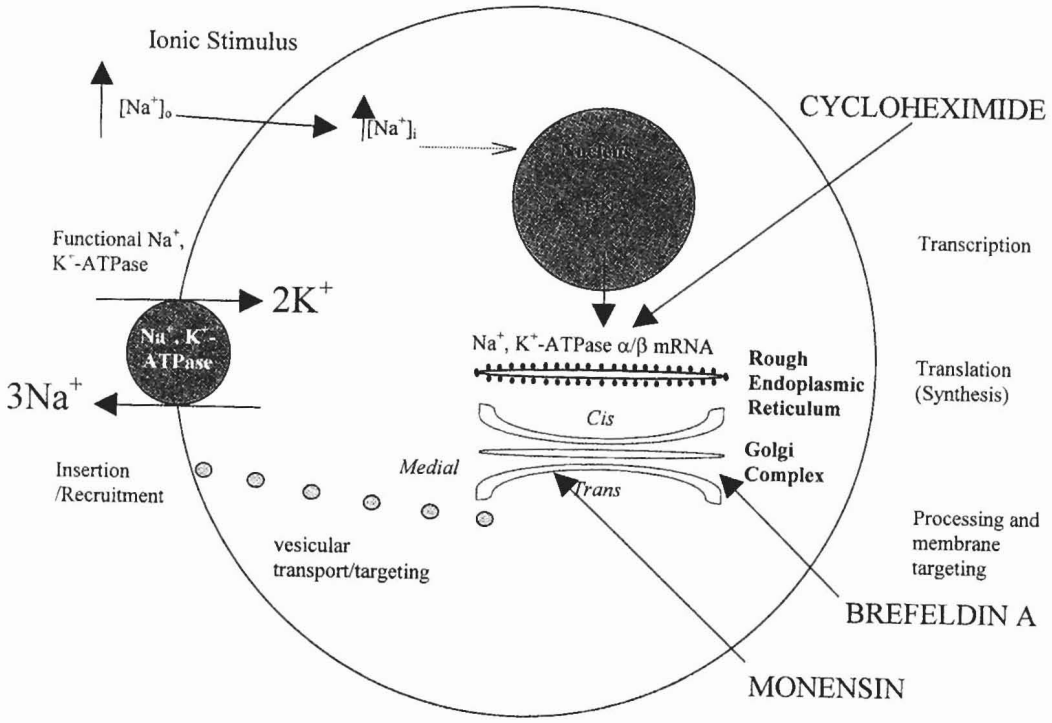


Fig. 1. Schematic representation of the intracellular sites of drug action. Brefeldin A, cycloheximide and monensin were used in the ^3H-ouabain binding studies. Each drug has a different and specific target in the cell. Brefeldin A (which was also used in the confocal studies) blocks protein and glycoprotein transport from the endoplasmic reticulum (ER) to the Golgi complex and redistributes Golgi membranes into the ER, dilating the nuclear envelope which is continuous with the ER membranes (Misumi et al., 1986; Fujiwara et al., 1988; Doms and Yewdell, 1989; Lippincott-Schwartz et al., 1989, 1990). Cycloheximide prevents protein synthesis in chondrocytes by blocking the translocation reaction on ER ribosomes (Kimura et al., 1981). Monensin blocks protein secretion at the level of the trans-Golgi network (Rosa et al., 1992; Qiu et al., 1995).

monoclonal antibodies raised against the catalytic α isoforms) and to study the distribution of the catalytic α subunits of the Na^+, K^+-ATPase in normal and Brefeldin A treated chondrocytes.

Materials and methods

Chemicals and antibodies

All chemicals were A.C.S. or Molecular Biology grade and purchased from Sigma/Aldrich (Poole, Dorset, UK), and Bio-Rad (Hemel Hempstead, UK). ^{3}H-ouabain was purchased from Amersham International (Amersham, Bucks., UK). Tissue culture media (Dulbecco's modified Eagle's medium, DMEM), phosphate buffered saline (PBS) tablets and the inhibitors Brefeldin A, cycloheximide and monensin were also obtained from Sigma. Brefeldin A was stored at -20 °C in methanol as a 10mg/ml stock. The monoclonal antibody against Na^+, K^+-ATPase α3 isoform (clone XVIF9-G10) was obtained from Affinity Bio-Reagents (Neshanic Station, New Jersey, USA) through Cambridge BioScience (Cambridge, UK). Monoclonal antibodies against Na^+, K^+-ATPase α1 and α2 isoforms were obtained from Dr. Douglas M. Fambrough (Johns Hopkins University, Baltimore, USA) and Dr. Kathleen J. Sweadner (Harvard University, Boston, USA) respectively. Antibodies against resident proteins of the endoplasmic reticulum were obtained from Dr. Daniel Louvard (Institute Pasteur, Paris, France). The suitability of the α-specific antibodies has been previously evaluated by Western blotting and immunofluorescence laser confocal microscopy (Mobasheri et al., 1997b).

Chondrocyte isolation

Bovine metacarpal-phalangeal joints from the front feet of 18-24 month old steers were obtained fresh (within 2h of slaughter) from a local abattoir. The joints were washed, skinned, sprayed with 70% ethanol (v:v with water) and opened under sterile conditions in a tissue culture hood. Cartilage shavings were cut from the joint surface and placed in sterile DMEM containing different sodium concentrations (120 and 220mM [Na^+]). Chondrocytes were isolated by bacterial collagenase digestion of the cartilage explants using Sigma type I collagenase (from *Clostridium histolyticum*, 100 collagen digestion units ml^{-1}). Sterile DMEM containing 120mM or 220mM Na^+ was used for the isolation and supplemented with 25 units ml^{-1} penicillin-streptomycin (100μg ml^{-1}) solution for 18h at 37 °C in the presence and absence of drugs. The following drug concentrations were used: cycloheximide (100μg ml^{-1}); Brefeldin A (5μg ml^{-1}); monensin (10μg ml^{-1}). The digested cartilage resulted in a cell suspension which was passed through a coarse metal mesh to remove any undigested pieces of cartilage and subsequently filtered through a 70 μm Nylon membrane filter insert (Falcon) into a 50 ml centrifuge tube (Falcon). The chondrocytes were then spun down and washed twice with fresh DMEM to remove the collagenase, and the final cell pellet was resuspended in fresh DMEM. Cell numbers were calculated by using the vital dye Trypan blue on an improved Neubauer haemocytometer and Olympus BH-2 light microscope. Chondrocyte viability was determined after isolation and during the experimental procedures in the presence and absence of the drugs. Viability was normally greater than 93% but data are only presented from experiments performed when viability was greater than 95%.

^{3}H-Ouabain binding

Plasma membrane Na^+, K^+-ATPase density was measured by ^{3}H-ouabain binding according to Erdmann (1982), in a K^+ free artificial medium containing all the components present in DMEM except amino acids and vitamins. Since K^+ competes with ouabain for binding to the α subunit of the Na^+, K^+-ATPase, to achieve equilibrium binding quickly, K^+ must be removed from the extracellular medium prior to incubation with ^{3}H-ouabain. Thus, chondrocytes were washed 3x in K^+ free medium to remove the K^+. Sodium concentrations of 120 and 220mM were maintained in the K^+ free medium. The determination of Na^+, K^+-ATPase pump density was performed in this solution containing 4-8kBq/ml of the specific inhibitor ^{3}H-ouabain which binds to all known α isoforms (Sweadner, 1995). Non-specific ouabain binding was determined by the addition of excess non-radioactive ouabain (10^{-4} M) which saturates the specific sites leaving only the non-specific component (Erdmann, 1982) which was then determined by ^{3}H-ouabain addition. After incubating for 2h at 37 °C, the cells were washed 3x with ice-cold unlabelled K^+ free medium before lysing in 0.5% v:v Triton X-100 in deionized water. Control time course experiments showed that under these conditions, complete binding occurred within 60-90 min (data not shown). One half of the final Triton X-100 lysate was used for the determination of radioactivity by scintilla-tion counting, the other half was used for DNA assays. By taking a value 7.7pg DNA/bovine chondrocyte (Kim et al., 1988; Mobasheri et al., 1997a,b; Mobasheri, 1998), specific ^{3}H-ouabain binding/chondrocyte (Na^+, K^+-ATPase density/cell) was determined. Data are presented as Na^+, K^+-ATPase density/chondrocyte.

Cell fixation and Immunolabelling

Isolated chondrocytes were fixed in 3.7% para-formaldehyde in PBS for 10 minutes at room temperature. After removal of the fixative the cells were washed 4x with PBS and permeabilized by incubation with 0.5% Triton-X100 in PBS for 5 minutes at room temperature. Subsequently, the cells were washed 3x with PBS. Non-specific binding was blocked by incubating the cells with PBS containing 10% normal goat serum (NGS) overnight at 4 °C (up to 16h). The

primary monoclonal Na^+, K^+-ATPase α isoform specific monoclonal antibodies were diluted with PBS containing 1% NGS (2h at room temperature). Polyclonal rabbit antiserum against resident proteins of the endoplasmic reticulum (ER) was diluted in PBS containing 2% NGS. After incubation with the primary α-isoform specific monoclonal antibodies, the cells were washed 4x in PBS, and incubated with FITC-conjugated anti-mouse IgG (Sigma, diluted 1:128) in PBS containing 1% NGS (2h at room temperature). Cells labelled with antibodies against the ER were probed with TRITC-conjugated anti-rabbit IgG (Sigma, diluted 1:160) in PBS containing 1% NGS (2h at room temperature). The cells were then washed 4x with PBS and mounted in non-fluorescent mounting media (Hydromount®, National Diagnostics, USA).

Confocal image acquisition and analysis

Chondrocytes probed with monoclonal antibodies against the catalytic α isoforms in the presence and absence of Brefeldin A were examined using a Bio-Rad MRC 600 confocal laser-scanning microscope attached to a Nikon Diaphot inverted microscope with an oil immersion objective (x100). For excitation of FITC and TRITC, the nm line of an argon-ion laser was used. With the pin-hole filter combination, laser power setting and FITC/TRITC filters, 3-D optical images were recorded with intervals of approximately 0.5-1.0 μm in the z direction. The images were median filtered (3x3) to remove noise and saved in a 512x512 pixel or 256x256 pixel TIFF format (tagged image file format). Chondrocytes exhibiting high fluorescence signals were arbitrarily chosen for image analysis. All confocal images shown are in the z direction.

Visualisation of the endoplasmic reticulum before and after Brefeldin A treatment by fluorescence microscopy

Antibodies against resident proteins of the endoplasmic reticulum (ER) were used to visualise this organelle in the presence or absence of Brefeldin A (5μg ml^{-1}, 18h). Briefly, paraformaldehyde fixed, Triton X-100 permeabilized chondrocytes were probed with a rabbit antiserum against four resident ER polypeptides (Louvard et al., 1982) as described earlier. Visualisation was by conventional immunofluorescence microscopy using a Leica fluorescence microscope with appropriate FITC and TRITC filters. Fluorescence micrographs were taken using Kodak® Professional colour reversal film (400 ASA, exposures 1-4 min). The developed slides were scanned using a Nikon® CoolScan® slide scanner, saved in the tagged image file format (TIFF) on an Apple Power Macintosh computer and printed on a Codonics® photographic printer.

Results

Upregulation of Na^+, K^+-ATPase in isolated chondrocytes in response to elevated [Na^+]

Chondrocytes isolated overnight in DMEM culture medium containing 120mM [Na^+] expressed 117812 pump sites/cell (±2131 SEM, n=4); in 220mM [Na^+]

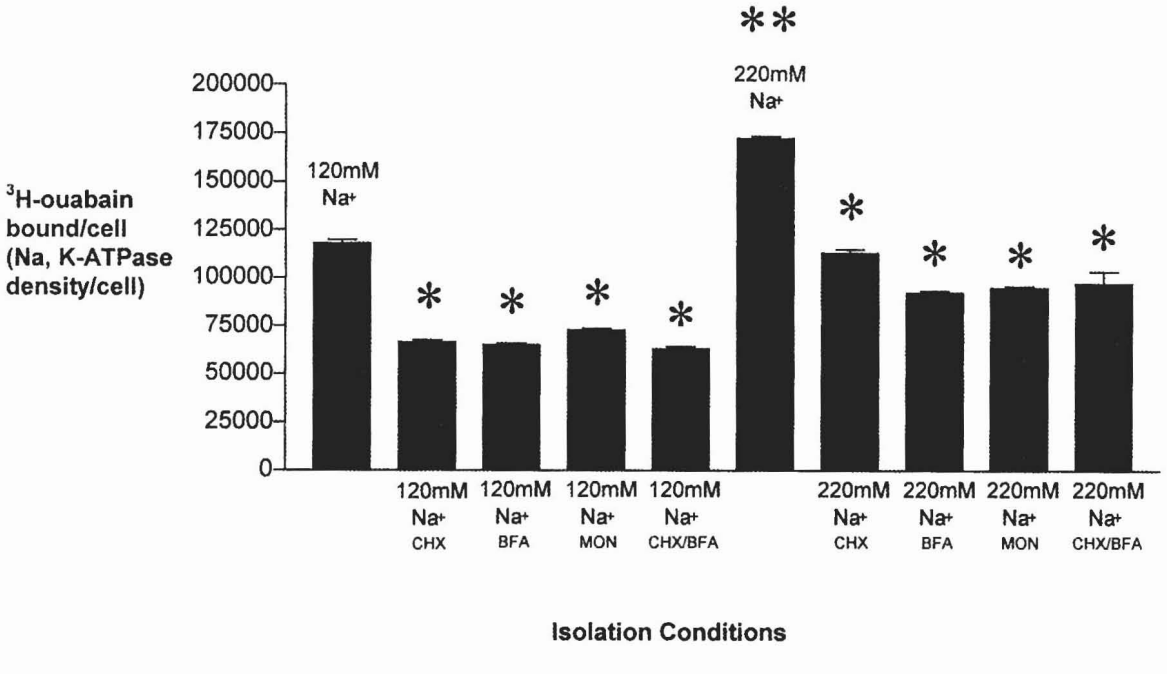


Fig. 2. Summary of ^{3}H-ouabain binding experiments using cycloheximide, Brefeldin A and monensin. CHX: cycloheximide; BFA: Brefeldin A; MON: monensin; CHX/BFA: cycloheximide and Brefeldin A combined. All these drugs blocked the upregulation of the Na^+, K^+-ATPase density over the period of cell isolation (18h). The Na^+, K^+-ATPase densities of drug treated cells fell below that of control, untreated cells, suggesting that membrane recycling and Na^+, K^+-ATPase degradation was unaffected by the drugs. *: $p<0.001$ (drug induced inhibition); **: $p<0.05$ (Na^+ induced upregulation)

pump density was upregulated to 172180 sites/cell (±1262 SEM, n=4) (Fig. 2). Pump density consistently increased with increasing [Na^+] and every ^{3}H-ouabain binding experiment performed showed a clear up-regulation in Na^+, K^+-ATPase density. In the presence of the protein synthesis inhibitor cycloheximide Na^+, K^+-ATPase density of chondrocytes isolated in 120 and 220mM Na^+ was significantly reduced; chondrocytes isolated overnight in DMEM culture medium containing 120mM [Na^+] and 100μg ml^{-1} cycloheximide expressed 66636 pump sites/cell (±963 SEM, n=3). Chondrocytes in DMEM culture medium containing 220mM [Na^+] and 100μg/ml cycloheximide expressed 112674 pump sites/cell (±2316 SEM, n=3). In the presence of the vesicular transport inhibitor Brefeldin A Na^+, K^+-ATPase density was reduced in a similar fashion by 44-46% (in 120mM Na^+ and 120mM Na^+ respectively). The carboxylic *trans*-Golgi secretion inhibitor monensin, had a similar inhibiting effect; Na^+, K^+-ATPase density fell by 38% in 120mM Na^+ and 45% in 220mM Na^+. Na^+, K^+-ATPase density (^{3}H-ouabain bound/cell) actually decreased in the cells treated with Brefeldin A and monensin. Since ^{3}H-ouabain only binds to the α subunit of active Na^+, K^+-ATPase pumps at the plasma membrane, it does not reveal information about intracellular pump turnover. For this purpose, monoclonal antibodies against the catalytic α subunits of Na^+, K^+-ATPase were used to probe paraformaldehyde fixed,

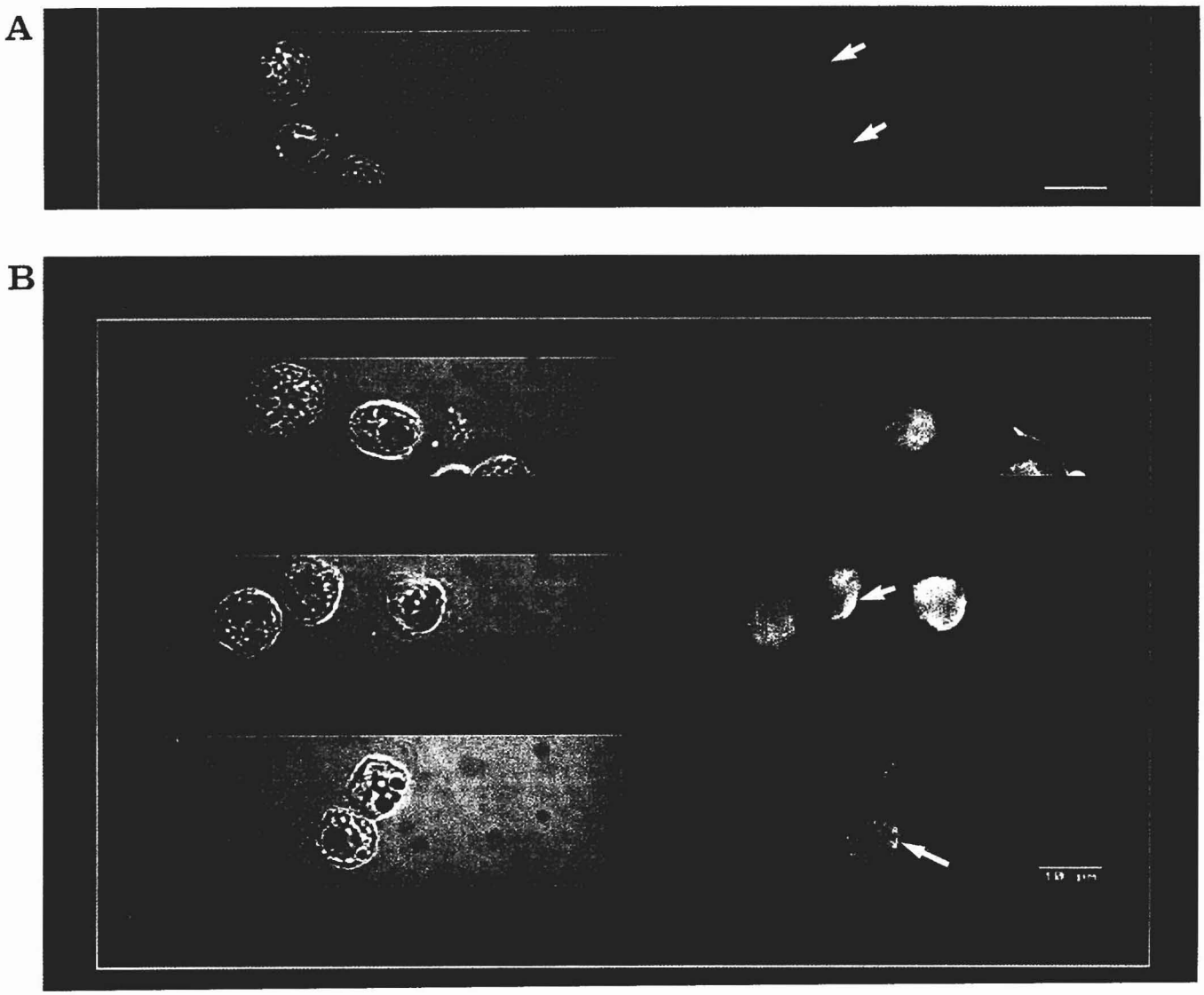
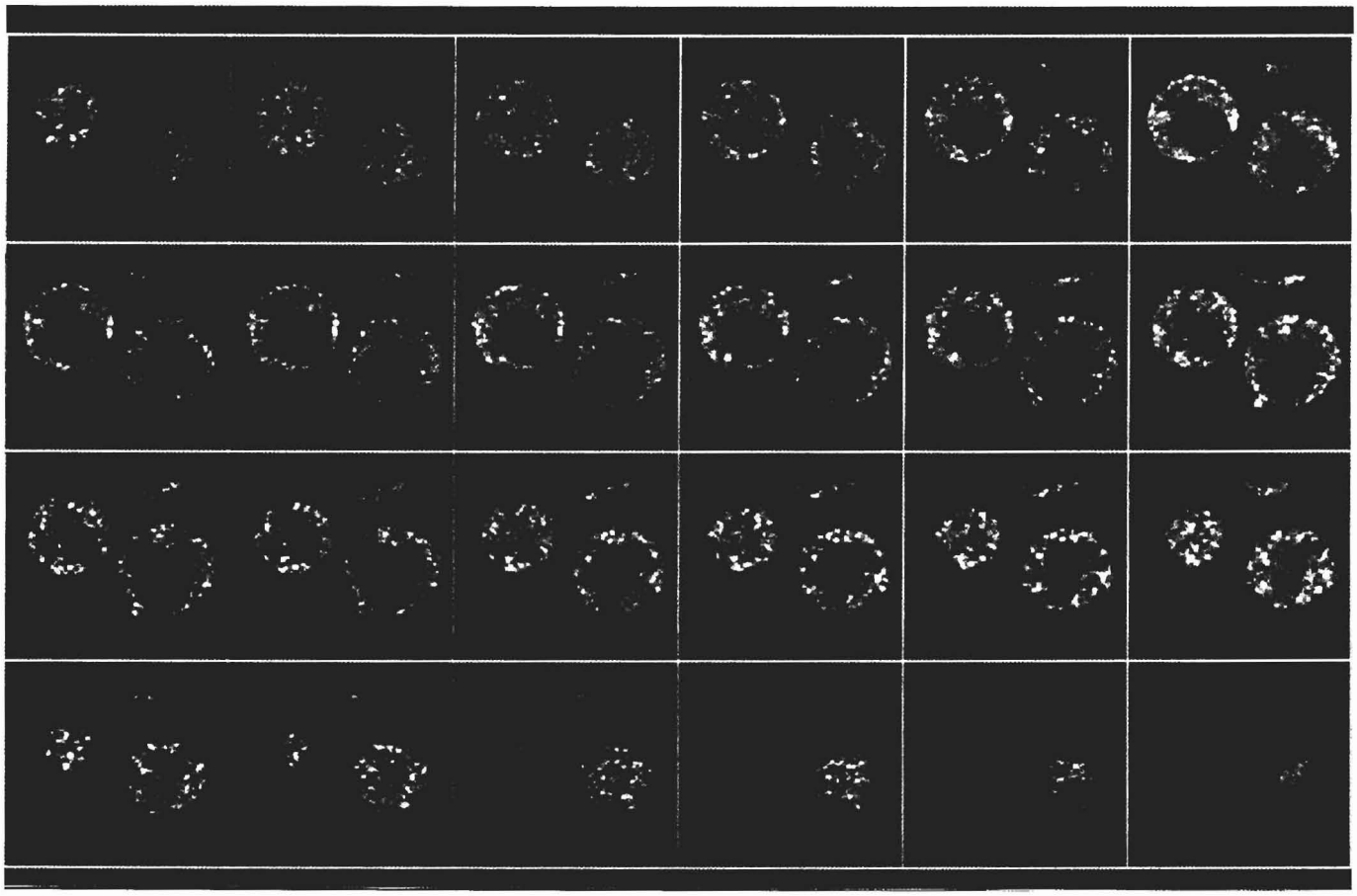


Fig. 3. Confocal reconstructions of control chondrocytes probed only with secondary FITC conjugated anti-mouse IgG **(A)** and chondrocytes probed with primary anti Na^+, K^+-ATPase α1 isoform specific mouse monoclonal and secondary FITC conjugated anti-mouse IgG **(B)**. Note the low background autofluorescence in control chondrocytes (A). Bar represents 10μm (approx.).

A



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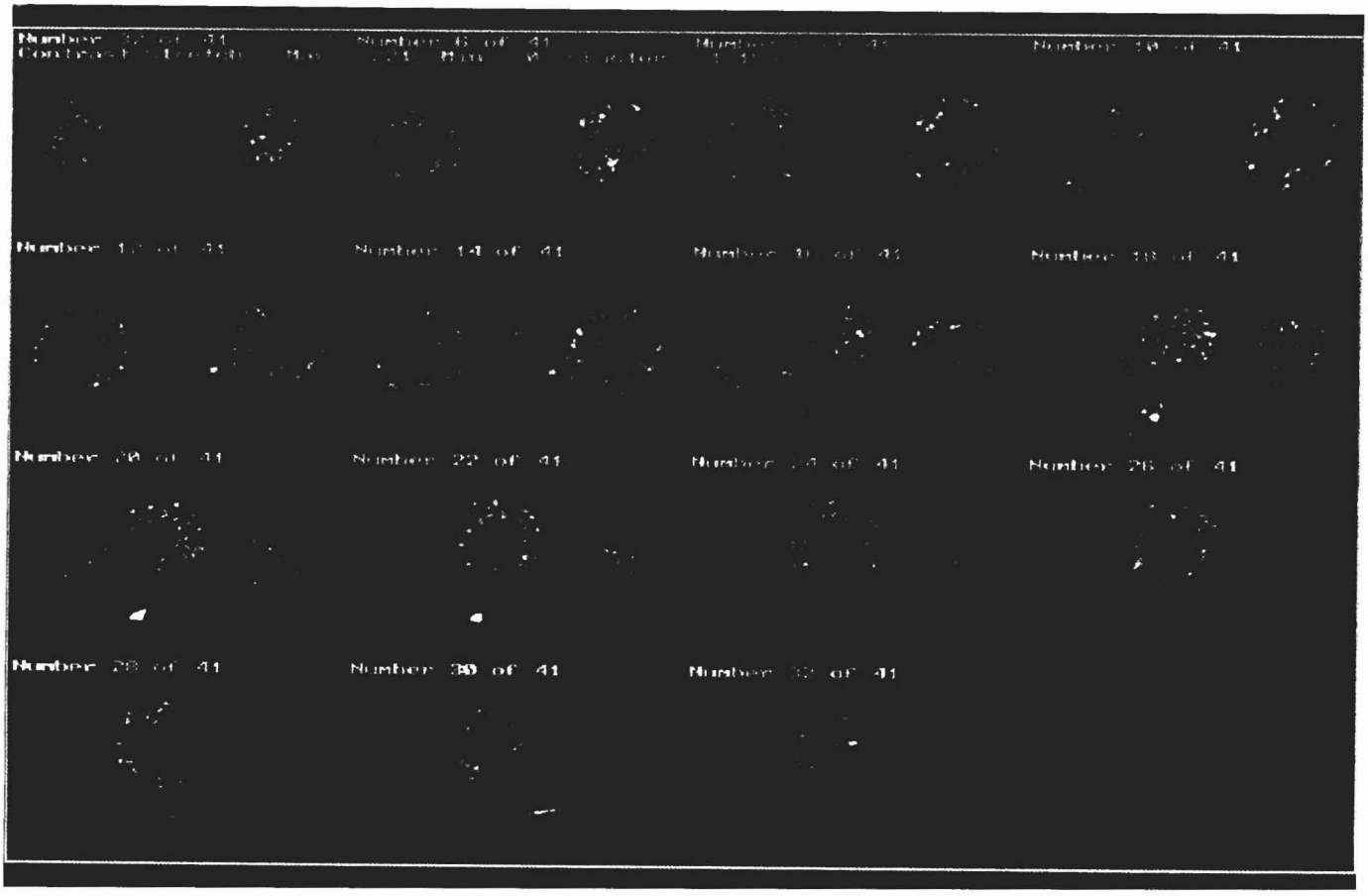

Fig. 4. A. Optical sections of chondrocytes probed with monoclonal antibodies against the α1 isoform of the Na+, K+-ATPase. **B.** Optical sections of chondrocytes probed with monoclonal antibodies against the α3 isoform of the Na+, K+-ATPase. In both cases the peripheral (plasma membrane) and intracellular pools of the α1 and α3 isoform subunits in freshly isolated cells are apparent.

Triton X-100 permeabilized chondrocytes to reveal the intracellular pools of the Na+, K+-ATPase and study the effect of Brefeldin A on α isoform distribution within the chondrocytes.

The effect of Brefeldin A on the intracellular pools of Na+, K+-ATPase α subunits and the endoplasmic reticulum

Immunofluorescence confocal laser scanning microscopy was used to localise Na+, K+-ATPase α subunits in chondrocytes isolated in the presence and absence of Brefeldin A which disrupts vesicular transport but does not interfere with protein synthesis (Misumi et al., 1986). The α1 isoform was found to be abundantly expressed in isolated chondrocytes (Figs. 3, 4A). In contrast the expression of the α3 isoform was lower in untreated chondrocytes (Fig 4B). If newly synthesised Na+, K+-ATPase α subunits were to appear following

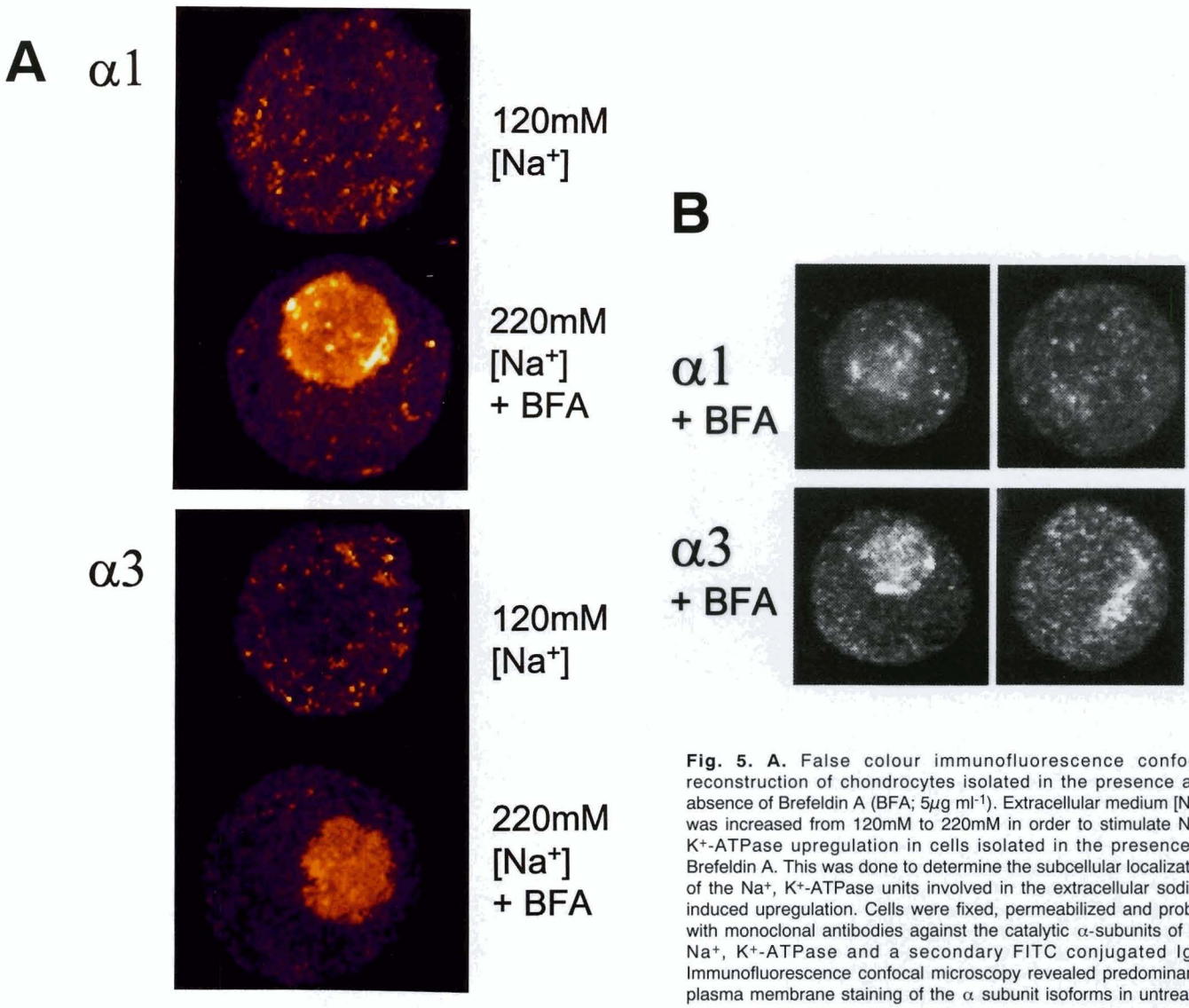

Fig. 5. A. False colour immunofluorescence confocal reconstruction of chondrocytes isolated in the presence and absence of Brefeldin A (BFA; 5μg ml-1). Extracellular medium [Na+] was increased from 120mM to 220mM in order to stimulate Na+, K+-ATPase upregulation in cells isolated in the presence of Brefeldin A. This was done to determine the subcellular localization of the Na+, K+-ATPase units involved in the extracellular sodium induced upregulation. Cells were fixed, permeabilized and probed with monoclonal antibodies against the catalytic α-subunits of the Na+, K+-ATPase and a secondary FITC conjugated IgG. Immunofluorescence confocal microscopy revealed predominantly plasma membrane staining of the α subunit isoforms in untreated cells. In contrast, BFA treated cells isolated in high [Na+] exhibited lower plasma membrane staining and intensely fluorescent intracellular staining. Most of the α-subunit immunostaining was in the nuclear envelope. The pattern of intracellular staining for each α isoform was different. The majority of the BFA treated cells (over 75%) exhibited a fully collapsed intracellular, peri-nuclear α subunit pool, while others exhibited partial collapse **(B)**. The fluorescence intensity of Brefeldin A treated chondrocytes probed with α1 and α3 specific antibodies was higher suggesting that these isoforms are preferentially upregulated in response to elevated extracellular [Na+]

ionic stimulation of chondrocytes, they would accumulate in the cell. The immunofluorescent staining of the α isoforms in untreated control cells isolated in 120mM [Na^+] appeared to be predominantly peripheral (plasma-lemmal) as expected. However, chondrocytes isolated in 220mM [Na^+] in the presence of Brefeldin A demon-strated large intracellular pools of catalytic α1 and α3 isoforms compared to chondrocytes isolated in 120mM [Na^+] in the absence of Brefeldin A (Fig. 5A). The plasma membrane immunostaining observed with untreated cells was reduced consistent with the reduced quantitative plasma membrane ^{3}H-ouabain binding (see Fig 2); intense fluorescent staining was observed in an intracellular juxta-nuclear compartment resembling the nuclear envelope (Fig 5A). The Na^+, K^+-ATPase α isoforms accumulated in the cell in response to elevated extracellular [Na^+] that was maintained for 18h. The intensity of intracellular staining was significantly higher than cell surface staining in these cells suggesting that α subunit proteins were being synthesised *de novo* in response to the ionic stimulus by accumulating within the cells.

The morphology of the ER in untreated cells was reticular and diffuse throughout most of the cytoplasm

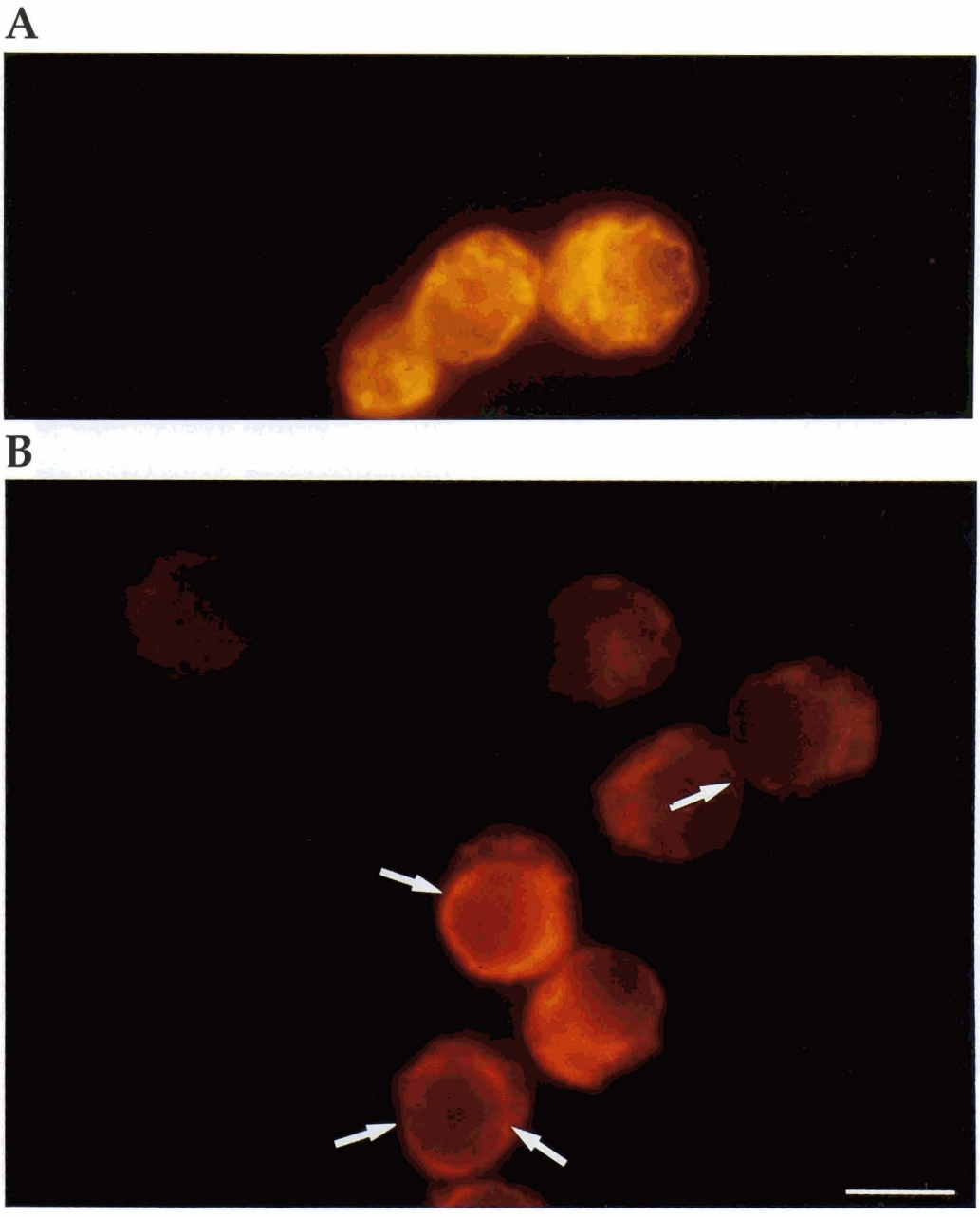

Fig. 6. The effect of Brefeldin A on the resident protein markers of the endoplasmic reticulum (ER) in isolated bovine articular chondrocytes. **A.** The cells shown were isolated as described in the Materials and Methods and following paraformaldehyde fixation and Triton X-100 permeabilization they were probed with polyclonal antibodies against resident proteins of the ER (Louvard et al., 1982). In normal chondrocytes, the ER appears to be an extensive reticular network pervading most of the cytoplasm. **B.** In chondrocytes treated with Brefeldin A (5μg ml^{-1}, 18h), the ER membrane collapse around the nucleus losing their reticular morphology (arrows indicate collapsed ER membranes). Bar represents 10μm (approx.).

(Fig. 6A). In Brefeldin A treated cells isolated in high [Na^+], the ER was fully dilated (Fig. 6B); in the same cells, the nuclear envelope appeared to be loaded with newly synthesised Na^+, K^+-ATPase α subunits (see Fig. 5). Although plasma membrane staining was still apparent, most of the immunostaining was in the intracellular juxta-nuclear compartment.

Discussion

In this study the abundance and turnover of the Na^+, K^+-ATPase of chondrocytes was examined to determine the molecular mechanisms involved in the long-term upregulation of the enzyme induced by elevated extracellular [Na^+]. Previous studies have demonstrated the presence of two catalytic α isoforms (α1>>α3) of the Na^+, K^+-ATPase in these cells (Mobasheri et al., 1997a,b). Here the contribution of these isoforms to the upregulation of cell surface Na^+, K^+-ATPase was further investigated in chondrocytes isolated in the presence and absence of various pharmacological agents.

The idea that the Na^+, K^+-ATPase is not a permanent cell surface resident was introduced over 20 years ago (Vaughan and Cook, 1972). The early experiments of Boardman and co-workers (1974) showed that regulation of Na^+, K^+-ATPase expression is under genetic control and many studies have since demonstrated that the expression of Na^+, K^+-ATPase is controlled at the pre- and post-translational levels (Taormino and Fambrough, 1990; McDonough et al., 1992; Fambrough et al., 1994). The Na^+, K^+-ATPase is in a state of constant molecular flux; it is synthesised in the ER, glycosylated in the Golgi apparatus, targeted to and inserted in the plasma membrane and subsequently internalised and degraded at rates that are tightly regulated by cells and are dependent on indirect hormonal effectors of the Na^+, K^+-ATPase and the intracellular and extracellular concentrations of Na^+ and K^+ respectively (Pollack et al., 1981; Wolitzky and Fambrough, 1986; Pressley, 1988; Ismail-Beigi et al., 1988; Brodie and Sampson, 1989). The α and ß subunits of the Na^+, K^+-ATPase are both glycoproteins; the α subunit has a monosaccharide attached to it (Pedemonte and Kaplan, 1992) and the ß subunit bears three N-linked oligosaccharides (Fambrough et al., 1994). Thus, newly synthesised Na^+, K^+-ATPase α and ß subunits must traverse the ER and Golgi complex in order to be correctly glycosylated before being targeted to the cell surface.

For the purpose of this discussion, long-term regulation of the Na^+, K^+-ATPase refers to the control of the plasma membrane density and cell surface abundance of Na^+, K^+-ATPase in chondrocytes. There are two mechanisms that can account for the increased abundance of a membrane resident protein such as the Na^+, K^+-ATPase: (I) *de novo* biosynthesis and decreased turnover of existing proteins, or (II) recruitment of existing proteins from an intracellular pool (Pressley, 1988). The most likely scenario is a combination of the two mechanisms depending on the nature and duration of the ionic or hormonal stimulation (Bowen and McDonough, 1987; Rossier et al., 1987; McDonough et al., 1992). Wolitzky and Fambrough (1986) have demonstrated that ion-concentration-dependent stimulation of the Na^+, K^+-ATPase is mediated, at least in part, by increased biosynthesis; their immunological experiments show that the plasma membrane upregulation of the Na^+, K^+-ATPase occurs mainly as a result of increased biosynthesis. Recruitment from inactive intracellular stores does not seem to account for the bulk of the upregulation, at least in cultured chick skeletal muscle cells (Wolitzky and Fambrough, 1986). Studies of Na^+, K^+-ATPase biosynthesis and regulation in chick sensory neurons have indicated 30% to 55% of the newly synthesised Na^+, K^+-ATPase does not appear on the cell surface but accumulates intracellularly, presumably in an inactive form (Tamkum and Fambrough, 1986). If this is true for all cells, arresting the movement of the newly synthesised Na^+, K^+-ATPase by adding pharmacological agents such as Brefeldin A should, theoretically, result in the over-accumulation of the pump components in an intracellular location. Brefeldin A has been shown to block the insertion of many membrane proteins into the plasma membrane. These include cell surface glycoproteins (Magner and Papagiannes, 1988; Qui et al., 1995), the insulin receptor (Kato et al., 1990), the epidermal growth factor (EGF) receptor (Gamou et al., 1988), the transferrin receptor (Schonhorn and Wessling-Resnick, 1994) and the immunoglobulin receptor (Apodaca et al., 1993). In the context of the present study, Brefeldin A has also been found to interfere with other plasma membrane transporters. Brefeldin A inhibits the synthesis and targeting of the GLUT2 isoform of the plasma membrane glucose transporter (Thorens et al., 1993) and blocks the insulin-induced recycling of the GLUT4 isoform between the plasma membrane and its intracellular pools (Lachaal et al., 1994; Bao et al., 1995). Thus far, there are no reports on the specific action of Brefeldin A on the Na^+, K^+-ATPase or any other ion transporting ATPase.

The plasma membrane abundance of the Na^+, K^+-ATPase is sensitive to the extracellular ionic environment of chondrocytes; Na^+, K^+-ATPase is upregulated in response to long-term exposure to increased extracellular [Na^+] (Mobasheri et al., 1997a,b; Mobasheri et al., 1998) and specific inhibitors of protein synthesis and vesicular transport block this upregulation. Chondrocytes treated with cycloheximide (a potent inhibitor of protein synthesis in these cells; Kimura et al., 1981) were unable to upregulate plasma membrane Na^+, K^+-ATPase. This suggests that *de novo* synthesis is the major component of the observed upregulation in control cells and an absolute requirement for physiological regulation of the Na^+, K^+-ATPase since the quantity of ^{3}H-ouabain bound to the cycloheximide treated cells was lower compared to control cells suggesting that Na^+, K^+-ATPase units are turning over in

the plasma membrane (presumably being internalised by endocytosis and degraded via the lysosomal-endosomal pathway). Protein synthesis is therefore required to maintain the cell surface Na^+, K^+-ATPase density in the absence of any stimuli for upregulation. The block induced by Brefeldin A treatment argues that Na^+, K^+-ATPase α subunit targeting and recruitment from intracellular membranes is also important. ^3H ouabain binding studies suggest that plasma membrane Na^+, K^+-ATPase abundance in tissue explants and isolated preparations of chondrocytes increases following long-term stimulation with elevated extracellular [Na^+] (Mobasheri, 1998; Mobasheri et al., 1997a,b). Chondrocytes treated with the Brefeldin A and monensin were unable to upregulate plasma membrane Na^+, K^+-ATPase. These drugs do not interfere with protein synthesis and since cells are unable to upregulate Na^+, K^+-ATPase sites at the plasma membrane, the biosynthesis of proteins including the Na^+, K^+-ATPase would be expected to continue unhindered in the presence of these drugs. Experiments using cycloheximide demonstrated that new synthesis is important, but closer examination of Brefeldin A treated cells by confocal microscopy revealed that newly synthesised α1 and α3 isoforms preferentially accumulate in response to elevated extracellular [Na^+]. The α3 isoform of the Na^+, K^+-ATPase has been shown to possess a relatively low affinity for intracellular Na^+ (Jewell and Lingrel, 1991). It is likely that in chondrocytes Na^+, K^+-ATPase pumps containing the α3 subunit isoform have a specialised role in regulating an optimal Na^+:K^+ ratio in face of large inward [Na^+] gradients resulting from changes in the extracellular matrix. Immunofluorescence microscopy also indicated that chronic treatment of chondrocytes with Brefeldin A results in the expected morphological collapse of the ER cisternae into a mixed membrane compartment adjacent to the nucleus and continuous with the nuclear envelope. In the same chondrocytes the newly synthesised Na^+, K^+-ATPase α-subunits were sequestered in this intra-cellular mixed membrane compartment and thus prevented from reaching the plasma membrane destination.

To conclude, the long-term regulation of the Na^+, K^+-ATPase depends upon Na^+, K^+-ATPase subunit synthesis and recruitment of new pump units to the plasma membrane. The Na^+, K^+-ATPase is constantly turning over in these cells that routinely face large inward Na^+ gradients resulting from physico-chemical changes in the extracellular matrix of cartilage. This gives the chondrocyte the ability to respond to changes in extracellular fluid and intracellular [Na^+]. The Na^+, K^+-ATPase plays an important role in the adaptation of the chondrocyte to its unusual ionic environment. If the synthesis, abundance and plasma membrane activity of the Na^+, K^+-ATPase is not precisely regulated, the low intracellular Na^+:K^+ ratio essential for enzyme activity and matrix synthesis cannot be maintained. As a result there may be deleterious changes to the physiological ionic homeostasis in the cell resulting in changes to the equilibrium between synthesis and degradation of cartilage matrix macromolecules (Urban and Bayliss, 1989; Urban et al., 1993). In the long-term, this may contribute to or exacerbate degenerative cartilage abnormalities.

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