

## Mediation of annexin 1 secretion by a probenecid-sensitive ABC-transporter in rat inflamed mucosa

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### Abstract

Annexin 1 is secreted by mammalian cells but lacks a leader signal sequence necessary to lead it to the classical secretory pathway via the endoplasmic reticulum. The mechanisms involved in the secretion of leaderless proteins remain uncertain. It has been suggested to involve membrane translocation via an ABC-transporter (ATP binding cassette). Using cultured inflamed mucosa from rectocolitis induced in rats, we studied if annexin 1 secretion followed the two main characteristics of ABC-transporter substrates: dependency on ATP hydrolysis and competitive inhibition by several other ABC-transporter substrates. Annexin 1 secretion is inhibited in a dose-dependent manner by two ATPase inhibitors. The inhibition reached  $63.2 \pm 3.2\%$ ,  $66.1 \pm 3.73\%$  and  $88.6 \pm 1.4\%$  in the presence of 2 mM vanadate, 0.5 and 1 mM pervanadate, respectively. The efflux of calcein, a known ABC-transporter substrate, is similarly inhibited by  $69.4 \pm 2.8\%$  in the presence of 1 mM pervanadate. Probenecid, an inhibitor of several ABC-transporters of the subfamily ABCC or MRP (multidrug resistant associated protein), also inhibited annexin 1 secretion in a dose-dependent manner. As compared to control, 10 mM probenecid reduced annexin 1 secretion by  $72 \pm 20\%$  and 20 mM by  $95.0 \pm 9\%$ . By contrast, annexin 1 secretion is not blocked by other inhibitors of MRP1 (indomethacin, MK571), MRP2 (ochratoxin A1 or MK571), MRP5 (trequinsin or sulfinpyrazone) or by verapamil, cyclosporin A or glyburide. Taken together, our results show that annexin 1 secretion appears to share the efflux properties of ABC-transporter substrates. © 2003 Elsevier Inc. All rights reserved.

**Keywords:** Annexin 1; Inflammation; Rectocolitis; Rat; Secretion; ABC-transporter

### 1. Introduction

Annexin 1 belongs to a family of calcium and phospholipid binding proteins which have been identified from primitive organisms such as hydra to vertebrates and plants [1]. Annexins are mainly intracellular proteins localized either free in the cytosol or associated with cellular membranes or cytoskeletal proteins [1]. However, the secretion of several annexins has been detected in certain tissues or cells under physiological or pathophysiological conditions

such as inflammation or cancer [2–9]. Nevertheless, annexins are not secreted through the classical exocytic pathway and the lack of a hydrophobic leader peptide prevents them from being targeted to the endoplasmic reticulum [5,10–12]. They actually seem to be exported from cells through a distinct secretory pathway which also carries several other cytosolic and leaderless proteins to the medium such as galectins, S100 proteins, interleukins (IL) 1 and 18, thioredoxin and FGF [5,6,13–16]. The release of leaderless proteins has long been a subject of debate, even if some of them have clearly identified extracellular functions and specific cell surface receptors. The problem was to know if they are released by an authentic physiological secretion or in consequence of plasma membrane disruption. Annexin 1 was shown to be truly secreted because of the abundance and selectivity of its release and its modulation by several drugs and by incubation temperatures [2,5,6]. Annexin 1 secretion occurs in the prostate, brain, epithelial cells, phagocytes, folliculo stellate cells, astrocytes and several cell lines [2,5,6,9,22–24]. Secreted

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**Abbreviations:** ABC, ATP binding cassette; Calcein-AM, calcein acetyloxymethyl ester; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4'-diisothiocyanostylbene-2,2'-disulfonic acid; FGF, fibroblast growth factor; IL, interleukin; LDH, lactate dehydrogenase; MDR, multidrug resistance protein; MRP, multidrug resistance related protein; SUR, sulfonylurea receptor; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

annexin 1 can regulate cell migration, growth or differentiation and has anti-inflammatory properties such as inhibiting the production of inflammatory mediators, the leukocyte extravasation from blood or the secretion of hypothalamo-pituitary hormones [9,12–24].

One of the proposed hypotheses concerning the secretion of these proteins is their efflux through ABC-transporters [12], a family of ubiquitous membrane proteins which are expressed in all cells. They efflux a great variety of substrates by ATP-dependent membrane translocation [25]. This hypothesis was first suggested because some procaryotic ABC-transporters export large leaderless proteins [12]. The conservation of such a transport system in eucaryotes was suggested knowing that peptides are conveyed by several mammalian or yeast ABC-transporters [12,26,27] and because some inhibitors of ABC-transporters were recently shown to block the secretion of two mammalian leaderless proteins: IL-1 $\beta$  and basic-FGF [13,14].

The aim of this study was to determine whether annexin 1 secretion involves an ABC-transporter by studying if it is both dependent on ATP hydrolysis and sensitive to inhibitors of ABC-transporters. Some differences between this study and a recent report showing that annexin 1 secretion is inhibited by glyburide will be discussed [24].

This study was performed using fresh mucosa from rectum of rats experimentally inflamed by trinitrobenzenesulfonic acid. Indeed, these tissues were shown to actively secrete annexin 1 allowing the examination of its modulation [6].

## 2. Materials and method

### 2.1. Chemicals and products

All chemicals were from Sigma except sulfinpyrazone (from ICN), calcein-AM (calcein acetyloxymethyl ester, from Molecular probes) and MK571 which was a generous gift from Dr. A.W. Ford-Hutchinson (Merck-Frost). RPMI 1640, was purchased from Eurobio.

### 2.2. Experimental model to study annexin 1 secretion (see detailed procedure in [6])

A rectocolitis was induced in overnight fasted and anesthetized rats (Wistar, 250–350 g, Iffa-Credo) as previously described [28]. In short, 100 mg kg<sup>-1</sup> (1 mL kg<sup>-1</sup>) of trinitrobenzenesulfonic acid in 50% ethanol was injected in the rectal lumen through a catheter inserted 8 cm into the anus. One day later, the rats were sacrificed by cervical dislocation.

Inflamed mucosa was collected from the rectum and cut into small fragments. Extracellular annexin 1 was extracted from tissue fragments on ice, using four washes of 30 min in phosphate buffered saline (PBS: 150 mM NaCl, 2.5 mM

KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4). In order to solubilize extracellular annexin 1, the PBS was supplemented with 2 mM final EGTA except for the last wash. Mucosa was then incubated in RPMI 1640 with 20 mM Hepes, pH 7.4, for 30 min at 37°. Tissue fragments were finally adjusted to 2 mM EGTA, rapidly cooled to 0° and further incubated on ice for 30 min. In these conditions, protein secretion occurred in RPMI at 37° but not during initial and final incubations at 0° [6]. Tissues were separated from supernatants and were homogenized on ice in a lysis buffer. Cell viability was measured spectrophotometrically by detecting lactate dehydrogenase (LDH) activity in aliquots of fresh supernatants and homogenates [6].

### 2.3. Modulation of annexin 1 secretion

The effect of different inhibitors of ABC-transporter effluxes were investigated on annexin 1 secretion. They were added to the last wash in PBS and to RPMI medium: probenecid at the concentrations of 1, 5, 10 and 20 mM, MK571 at 10, 30 and 50  $\mu$ M, verapamil at 60, 200 and 600  $\mu$ M, cyclosporin A at 1 and  $\mu$ M, glyburide (glibenclamide) at 1, 10, 100 and 200  $\mu$ M, trequinsin at 0.1, 1 and 10  $\mu$ M, sulfinpyrazone at 0.1, 1 and 2.5 mM, ochratoxin A at 5, 50 and 500  $\mu$ M, indomethacin at 2, 10 and 50  $\mu$ M, DIDS or 4,4'-diisothiocyanostylbene-2,2'-disulfonic acid at 100 and 500  $\mu$ M, vanadate (*ortho*-vanadate) at 0.5, 2 and 5 mM and pervanadate at 0.5 and 1 mM. For the use of vanadate or pervanadate which inhibit ATPases, RPMI medium was replaced by a glucose-free medium (PBS containing essential amino acids) in order to reduce endogenous production of ATP. Pervanadate was produced from 20 mM vanadate in PBS by adding an equimolar amount of H<sub>2</sub>O<sub>2</sub> and incubating the solution for 20 min at 20° [29]. Except for vanadate, none of the drugs used were toxic to the mucosa, resulting in unchanged low LDH release into the medium (below 10%). In these conditions, the annexin 1 released into the medium was shown to be truly secreted [6]. In four out of six experiments performed vanadate showed a clear cytotoxic action on inflamed mucosa at either 2 or 5 mM, with a release of LDH to the medium superior to 16%. By contrast, the two other experiments showed no cell toxicity of vanadate and were included in results.

### 2.4. Immuno-detection of annexin 1 secretion (detailed description in [6])

Supernatant proteins were precipitated with 10% trichloroacetic acid on ice and centrifuged for 10 min at 20,000 g. Supernatant precipitates and homogenates were equilibrated in Laemmli's sample buffer, were subjected to SDS-PAGE [30] and separated proteins were transferred onto nitrocellulose membranes. The samples loaded on the 10% acrylamide gels contained 10  $\mu$ g of homogenate proteins and supernatant volumes corresponding to what was released by 50  $\mu$ g of tissue proteins. Annexin 1

expression was detected by immunoblotting using rabbit polyclonal antibody, ECL chemiluminescence and fluorography using Hyperfilm MP (Amersham Pharmacia). The rabbit polyclonal antibodies used were purified on an annexin 1 affinity column. These antibodies were previously characterized as monospecific to this protein by immunoblotting experiments and by immunohistochemistry [6,18]. Densitometric analysis of annexin 1 secretion was performed by scanning the immunoblotting films using the Gel Doc system (Bio-Rad) and calculating the percent of extracellular annexin 1 vs. the total expression of annexin 1 (tissue + supernatant). An internal standard (St) of tissue homogenates was loaded on gels containing samples of either homogenates or supernatants. This permitted us to compare annexin 1 expression in these different samples and to calculate the percentage of annexin 1 secretion from densitometric analysis.

### 2.5. Effluxes of calcein from inflamed mucosa

The effects of different chemicals on calcein release were tested in inflamed mucosa according to Hamilton *et al.* [31] but with little modifications to follow similar conditions as those used to study annexin 1 secretion. After the four initial washing steps in PBS, tissues were subjected to two consecutive incubation at 37° in PBSA (PBS supplemented with 0.63 mM CaCl<sub>2</sub>, 0.74 mM MgSO<sub>4</sub>, 5.3 mM glucose, 2 mM L-glutamine and 2 mM essential amino acids), to permit fluorescence measurement. First, tissues were incubated for 30 min with 1 µM calcein-AM to insure an equivalent uptake of the chemical. After that, the media were replaced by fresh PBSA without calcein-AM but in the presence or absence of either 1 mM pervanadate, 1 or 20 mM probenecid or 10 µM indomethacin. Tissues were then incubated for 1 hr 30 min to allow both significant calcein efflux and inhibitor action. Samples were then made up to 2 mM EDTA and incubated for 30 min on ice. After that, tissues and supernatants were separated. Tissues were homogenized as previously described and fluorescence of calcein was measured in supernatants (secreted calcein) and in homogenates using a fluorometer (LS 50 B, Perkin Elmer) at excitation/emission wavelengths of 485/530 nm. The fluorescence of tissues was corrected for autofluorescence of control tissues incubated without AM-calcein. Protein concentrations in homogenates were measured according to Bradford [32] and the fluorescence was calculated as units of fluorescence per milligram of tissue proteins in homogenates and in the corresponding supernatants. The percent of calcein secretion were then calculated from supernatant fluorescence vs. total fluorescence (tissue + supernatant).

### 2.6. Statistical analysis

All experiments were performed with six samples in absence of significant inhibition and with at least eight

samples to statistically assess inhibitory effects on secretions. Data are presented as means ± standard deviations. Statistical significance was assessed using ANOVA and determined as the 99.9% confidence limits.

### 2.7. RT-PCR detection of ABC-transporters mRNA

RT-PCR were performed using mouse tissues because ABCA1, MRP1, MRP2, MRP5 cDNA were already cloned in this species but not in rat. RNA were isolated from 100 mg of inflamed mucosa by thiocyanate–phenol–chloroform extraction using Extract-all (Eurobio). RNA from lung or liver were also isolated to be used as positive controls during RT-PCR. For each sample the mRNA of 5 µg RNA were reversed transcribed using M-MLV reverse transcriptase RNase H minus, point mutant (Promega) and oligo dT in a final volume of 20 µL. PCR was carried out in a 50 µL final volume using 0.25 U of Taq-Gold (Applied Biosystem), 2.5 mM MgCl<sub>2</sub> and either 1 µL of cDNA as a template or 1 µL of H<sub>2</sub>O for negative controls devoid of cDNA. The primers used were designed to specifically amplify mouse ABC-transporters and β-actin. For ABCA1, PCR were optimized at an annealing temperature of 65° and 45× cycles (30 s at 95°, 30 s at 65° and 1.5 min at 72°) using sense primer GTGAGCGCTGGCCTGCTGGTGGTC and antisens primer CCAGCCGGGCTTCAGACACGTGCTTCC to obtained a final amplification fragment of 1500 bp. For MRP1 sense and antisens primer were, respectively, AGGTGGCCCACATGAAGAGCAAAGAC and CACTGCTGAGAGGGGATCATCGAAGAGG, the annealing temperature of 60° and the final amplification fragment of 923 bp. For MRP2 sense and antisens primers were, respectively, ATGACTGTACGGACAACCATCA and CCAGAACGACAATCTCATCCAC, the annealing temperature of 60°, and the amplified fragment of 1335 bp length. For MRP5 sense and antisens primers were, respectively, CTCCTCCCACTCCAGTATACAGAACTCG and ATGCATCTGCCGCCCATCCACAA, the annealing temperature of 60°, and the amplified fragment of 1359 bp length. Finally, a fragment of 234 bp of β-actin were amplified using the sens and antisens primers AGACTTCGAGCAGGAGATGG and GCACTGTGTTGGCATAGAGG and at 54° of annealing temperature. The amplified fragments were separated in 1.2% agarose gel by electrophoresis in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA). They were verified to be the specific for their apparent size before and after endonuclease digestion.

## 3. Results and discussion

The experimental model of annexin 1 secretion was chosen in this study because we had previously shown that the protein release is abundant and corresponds to genuine secretion of 10–20% of total annexin 1 during 30 min incubation at 37° [6]. This was not obtained, in our

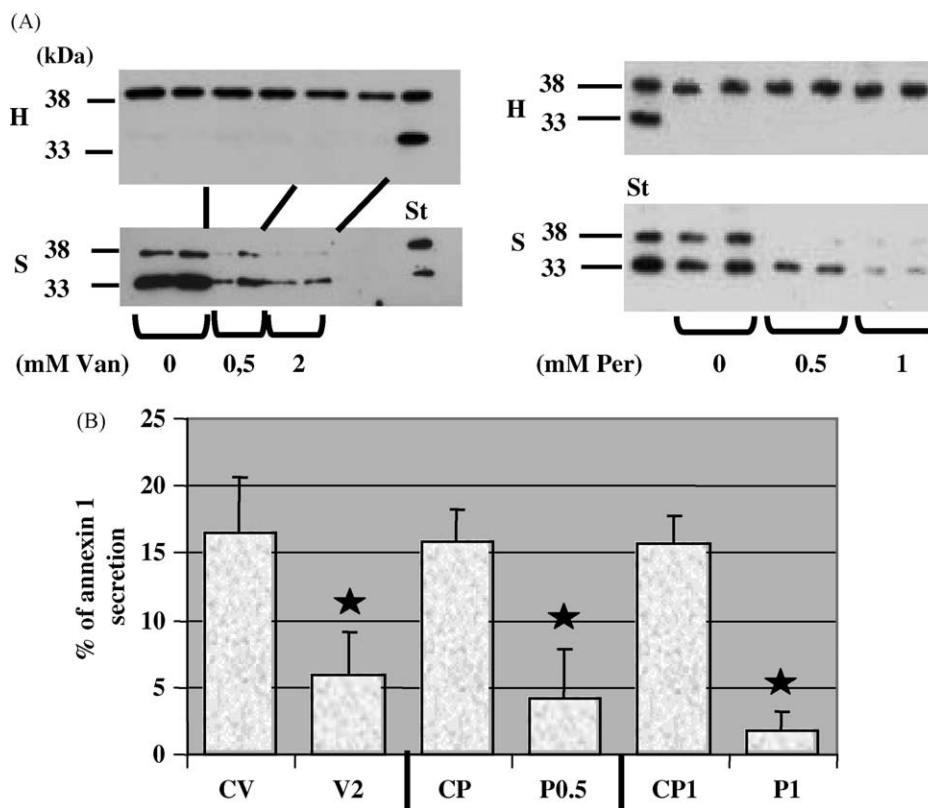


Fig. 1. ATP-dependence of annexin 1 secretion: (A) immunoblotting of tissue homogenates (H) and supernatants (S) of rat colonic mucosa after incubation with 0.5, or 2 mM vanadate (Van) or 0.5 or 1 mM pervanadate (Per). An internal standard (St) of tissue homogenates was loaded to compare annexin 1 expression in homogenates and supernatants. Molecular weights of detected annexin 1 are indicated in kDa. (B) Histogram showing the percent of annexin 1 secretion in the presence of 2 mM vanadate (V2) (two experiments,  $N = 4$ ), 0.5 or 1 mM pervanadate (P 0.5 and P 1) from 5 ( $N = 10$ ) or 4 separate ( $N = 8$ ) experiments. Respective control tissues for each treatment are indicated as CV ( $N = 4$ ), CP ( $N = 10$ ) and CP1 ( $N = 8$ ). Percent of annexin 1 secretion was calculated after densitometric scans of the intensity of annexin 1 detected in immunoblots of supernatants vs. supernatants plus tissue homogenates. Data are presented as mean  $\pm$  standard deviation. Statistical significance was determined by ANOVA using 99.9% (★) confidence limits when compared to control tissues.

hands, with other cell culture models. This strong secretion allowed a clear dissociation to be made between secretion and aspecific leaks from damaged cells. The inflamed mucosa used did not contain any viable epithelium but only neutrophils expressing and secreting annexin 1 [18].

Annexin 1 was detected in two forms in the immunoblots (Figs. 1 and 3). The native protein with an apparent molecular weight of 38 kDa in SDS-PAGE was the only form expressed in cells. Secreted annexin 1 was recovered either as the 38 kDa form or as a 33 kDa fragment resulting from extracellular proteolysis [6]. The ratio between the two forms of annexin 1 detected in supernatants was very variable from one experiment to another certainly because of differences in protease contents in the inflamed mucosa (6 and this study).

### 3.1. ATP-dependence of annexin 1 secretion

ATP-dependence of annexin 1 secretion was explored using two drugs, vanadate and pervanadate that are analogous to phosphate and that inhibit ATPases and phosphatases by blocking phosphoryl transfer to catalytic site [29].

Indeed, the efflux of any ABC-transporter substrate absolutely requires ATP binding and hydrolysis in the ATPase domains [25].

Annexin 1 secretion was dose-dependently inhibited by vanadate and pervanadate from 0.5 mM (Fig. 1A). Vanadate was first studied because it is a reference to characterize ATP dependency of ABC-transporters effluxes, especially *in vitro*, using membrane vesicles. However, only two experiments using vanadate were taken into account in this study because they showed both no cell toxicity and a clear inhibition of annexin 1 secretion (Fig. 1A and B). By contrast, four other experiments revealed a cytotoxic action of vanadate with 16% or more LDH release into supernatants which did not permit to study annexin 1 secretion (not shown). On the contrary, pervanadate which is more cell permeable than vanadate showed no cell cytotoxicity and significant inhibitions of annexin 1 secretion reaching  $66 \pm 3.7\%$  ( $N = 10$ ) at 0.5 mM and  $88.6 \pm 1.4\%$  ( $N = 8$ ) at 1 mM as compared to control secretion (Fig. 1A and B).

This study shows that annexin 1 secretion requires ATP hydrolysis which is the first indication of ATP-dependency of leaderless protein secretion in mammals. Pervanadate



also seems preferential to vanadate for studying ATP-dependent processes in whole cells.

### 3.2. Analysis of calcein efflux by inflamed mucosa

Calcein is a known substrate of ABC-transporters. After its uptake by cells, calcein-AM is cleaved by intracellular esterases to produce the fluorescent metabolite calcein. MDR1 or BSEP were reported to extrude calcein-AM but not calcein [33,34]. MRP1 was shown to mediate calcein efflux in several cancerous cells [31,33] because this release is inhibited by 10  $\mu$ M indomethacin a specific MRP1 inhibitor and by 1 mM probenecid which inhibits MRP1 but also MRP2, 4, 5 and 6 [31,33,35–38]. The measurement of calcein efflux was studied in our model to validate the functionality of ABC-transporters and the inhibitory action of 1 mM pervanadate (for ATP-dependency), 10  $\mu$ M indomethacin and 1 mM probenecid (inhibitors of MRP1 efflux) and 20 mM probenecid (inhibitor of annexin 1 secretion). This experiment showed that calcein extrusion is significantly reduced in the presence of 1 mM pervanadate by  $69.4 \pm 2.8\%$  as compared to control secretion (Fig. 2). This showed that ABC-transporter effluxes are functional and sensitive to pervanadate in our model. Indomethacin at 10  $\mu$ M and probenecid at 1 mM showed similar slight (9–12%) and non-significant inhibitions of calcein effluxes from inflamed mucosa as compared to controls. Their inhibitory action on calcein secretion is much lower than the one of pervanadate. This suggests that calcein efflux from rat inflamed mucosa involved only moderately MRP1 but mainly an additional ABC-transporter sensitive to pervanadate but not to probenecid or indomethacin. This is in accordance with the low level of MRP1 mRNA detected in rectal inflamed mucosa (Fig. 3). Finally, the presence of 20 mM of probenecid did not inhibit but rather stimulated calcein effluxes when com-

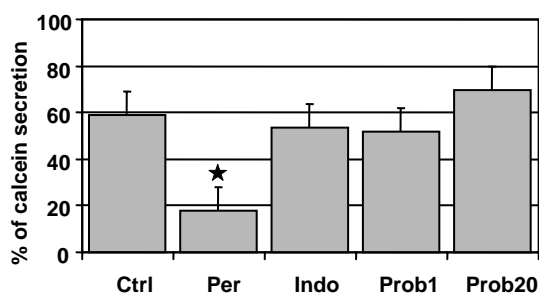


Fig. 2. Study of calcein secretion from inflamed mucosa of rat rectum. Tissues were incubated for 30 min in RPMI plus 1  $\mu$ M calcein-AM, then for 1 hr 30 min in fresh media without calcein-AM and in the absence of ABC-transporter inhibitor (ctrl, N = 8) or the presence of either 1 mM pervanadate (per, N = 8), 10  $\mu$ M indomethacin (indo, N = 8), 1 or 20 mM probenecid (prob1, N = 8 and prob20, N = 8). The percents of calcein secretion to the medium were calculated from the amount of supernatants fluorescence per milligram of tissue proteins vs. the total fluorescence (supernatants + homogenates) per milligram of tissue proteins. Statistical analysis was performed by ANOVA using 99.9% (★) confidence limits when compared to control tissues.

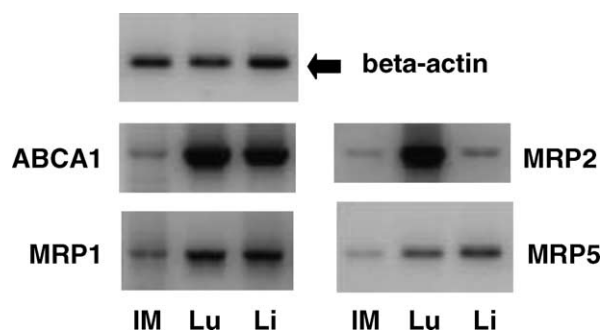


Fig. 3. RT-PCR analysis. The presence of the mRNA of ABCA1, MRP1, MRP2 and MRP5 and  $\beta$ -actin were detected in agarose gel after RT-PCR from mouse lung (Lu), liver (Li) and inflamed mucosa from rectum (IM).

pared to controls or tissues with 1 mM probenecid. Other experiments gave results in the same direction by showing that MRP2 effluxes could be potentiated by increasing probenecid concentrations [39,40]. This effect was shown to involve stimulation by probenecid of MRP2-ATPase activity [39].

### 3.3. Effect of ABC-transporter inhibitors on annexin 1 secretion

Most of the inhibitors of ABC-transporters are substrates and competitive inhibitors of these proteins. However, some drugs can bind and block their transporter to effluxes without being themselves transported [41]. Several known inhibitors of mammalian ABC-transporters were used in this study at concentration ranges previously reported for this effect. Probenecid is a non-selective inhibitor of several ABC-transporters and was clearly identified as a substrate and a competitive inhibitor of MRP1 [39]. Other inhibitors of several ABC-transporters were tested: MK571 inhibitor of MRP1 and 2, glyburide, inhibitor of ABCA1, CFTR (cystic fibrosis transmembrane conductance regulator or ABCB7), SUR1 (ABCB8) and SUR2 (ABCB9) (sulfonylurea receptors), DIDS an inhibitor of ABCC1 and verapamil and cyclosporine A, two inhibitors of MDR1 or 2/3 [13,31,33,35,41–45]. Among them, we identified by RT-PCR the expression of MRP1, 2 and 5 in inflamed mucosa but not those of MRP4 and 6 (Fig. 3). For this reason, the action of other known inhibitors of these transporters were tested in an attempt to relate annexin 1 release to some of them: indomethacin (inhibitor of MRP1), ochratoxin A (inhibitor of MRP2) and trequinsin and sulfinpyrazone (inhibitors of MRP5) [31,34,43]. Among the inhibitor tested only probenecid inhibits annexin 1 secretion from inflamed mucosa in a dose-dependent manner reaching of  $72 \pm 20\%$  inhibition at 10 mM and  $95 \pm 9\%$  at 20 mM (Fig. 4, results not shown for DIDS and cyclosporin A). The results suggest that MRP1, 2, 5, ABCC1, MDR1 or 3, ABCA1, CFTR nor SUR1 and 2 are not involved in annexin 1 secretion in inflamed mucosa.

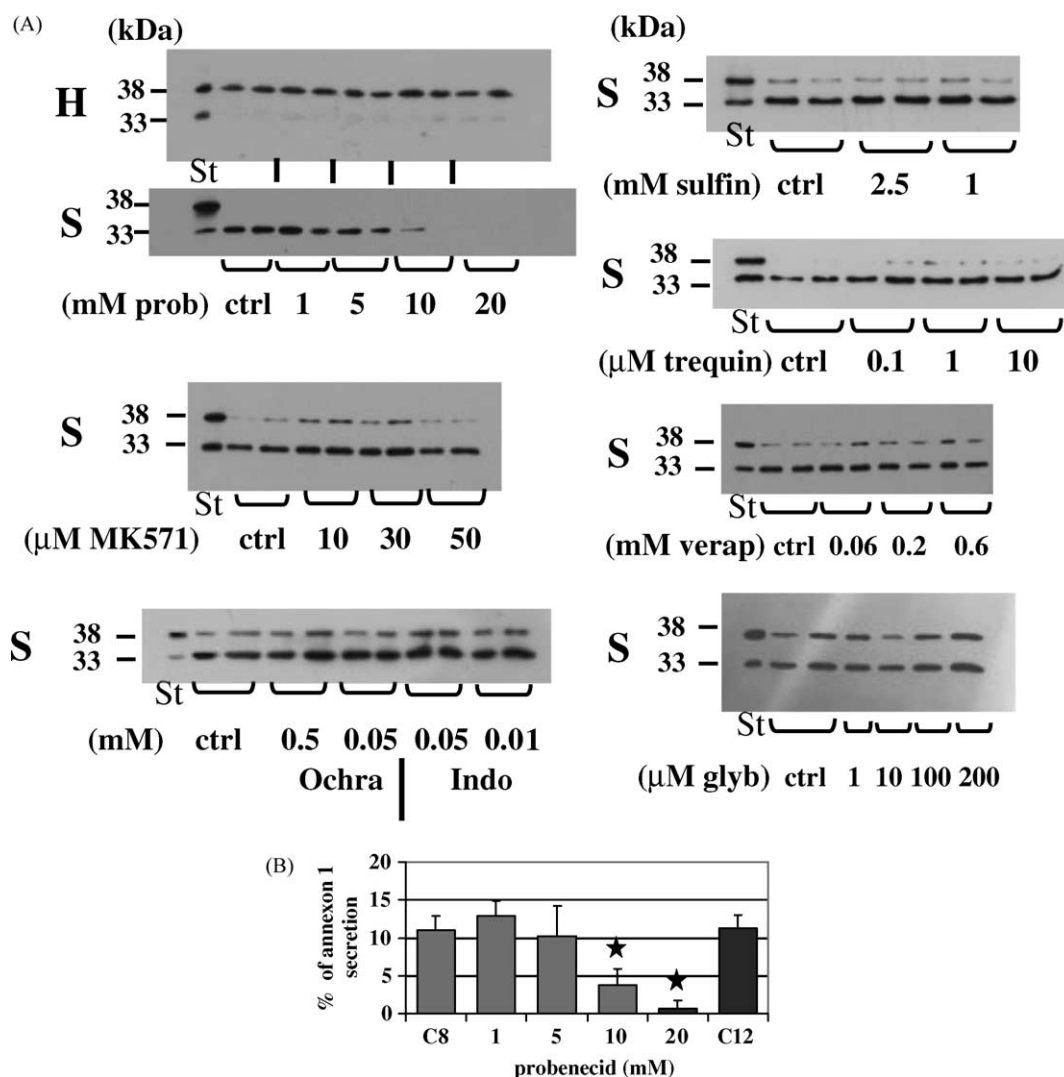


Fig. 4. Effect of ABC-transporter inhibitors in annexin 1 secretion. (A) Detection by immunoblotting experiments of the expression of annexin 1 in tissues homogenates (H) and supernatants (S) of rat colonic mucosa. Tissues were incubated in the absence of drugs (ctrl) or in the presence of different concentrations of probenecid (prob), MK571, ochratoxin (ochra), indomethacin (indo), sulfinpyrazone (sulfin), trequinsin (trequin), verapamil (verap), or glyburide (glyb). An internal standard (T) of tissue homogenates was loaded to compare annexin 1 expression in homogenates and supernatants and to calculate the percentage of annexin secretion. The molecular weights of the detected annexin 1 are indicated in kDa. The results shown are representative of three independent experiments. (B) Histogramical representation of the effect of probenecid on annexin 1 secretion performed in four (N = 8) or six (N = 12) separate experiments. Tissues were incubated in the absence (controls: C8 for N = 8, C12 for N = 12) or in the presence of probenecid at 1 mM (N = 8), 5 mM (N = 8), 10 mM (N = 8) or 20 mM (N = 12). Statistical analysis was determined by ANOVA using 99.9% (★) confidence limit when compared to control tissues.

A recent study reported that the release of annexin 1 by the pituitary folliculo-stellate cell line TtT/GF was inhibited by glyburide [24]. The authors suggested that ABCA1 is involved in annexin 1 release because it was also expressed in these cells and it co-localized with membrane associated annexin 1 by immunofluorescent labeling. By contrast, annexin 1 secretion by neutrophils from inflamed rectal mucosa appeared not to involve ABCA1, being insensitive to glyburide or DIDS. Interestingly, the annexin 1 release from TtT/GF cells is inhibited by glyburide when induced by 0.1 μM dexamethasone but not when induced by 56 mM of K<sup>+</sup> [24]. It should be noted that in our model, annexin 1 secretion was shown not to be induced by glucocorticoids [46]. This observation leads the possibility

that annexin 1 secretion involves distinct ABC-transporters from one cell type to another or from one secretion inducer to another. Interestingly, another experiment using antisense oligonucleotides of ABCA1 leads to the same hypothesis by showing that IL-1β secretion involved ABCA1 in macrophages but not in monocytes isolated from human blood [47].

Probenecid was reported to have other action in cells than inhibiting efflux by ABC-transporters. It also inhibits anion uptake by organic anion transporters which is not ATP-dependent [48] and renal oxidative metabolism, significantly reducing cellular ATP content from 1 mM [49]. This reduction should then inhibit effluxes by ABC-transporters. However, an active release of calcein is maintained

in our model during incubation in the presence of 20 mM probenecid for 1 hr 30 min whereas 1 mM pervanadate strongly inhibits it. This shows that, even in the presence of 20 mM probenecid, an intracellular pool of ATP remains available in inflamed mucosa to allow ATP-dependent mechanisms to occur. For these reasons, inhibition of annexin 1 secretion in our model should certainly not be attributed to ATP depletion by probenecid. The action of probenecid in oxidative metabolism could actually vary from one cell model to another. It could be related to the overall expression levels of ABC-transporters extruding probenecid and, in consequence, to the intracellular amount of the chemical. Probenecid is considered as a specific inhibitor of the ABCC subfamily. However, if it was reported not to inhibit MDR1 [50], its effect on many other ABC-transporters is very poorly documented.

The results from this study showed a clear and dose-dependent inhibition of annexin 1 secretion by vanadate, pervanadate and probenecid. The model of incubated inflamed mucosa appears then to be adequate to pharmacological characterization of annexin 1 secretion.

The secretion of basic-FGF is also inhibited by probenecid with a maximal effect at 1 or 5 mM for 2 hr incubation [14] and IL-1 $\beta$  secretion is inhibited by glyburide, DIDS and by sulfobromophthalein all three of which blocking ABCA1 transport [13]. The involvement of ABC-transporters in the secretion of leaderless proteins in mammals is supported today by two main observations. First, the release of b-FGF, IL-1 $\beta$  and annexin 1 are inhibited by distinct drugs whose common property is to be substrates and inhibitors of ABC-transporters. Second, annexin 1 secretion is shown in this study to be ATP-dependent.

A total of 48 ABC-transporters have been identified and sequenced in humans with a majority of them being also expressed in other mammals such as rodents (see <http://nutrigen.4t.com/humanabc.com> for description). Thirty-two of them are or could potentially be located in plasma membranes. Most of them remain to be characterized for their inhibitors and their endogenous and exogenous substrates to understand their respective functions. Part of this work would concern understanding ABC-transporter function in the secretion of leaderless proteins. It will include identifying which of them are involved in this process and determining whether they directly ensure membrane translocation of leaderless proteins, as prokaryotic transporters do, or whether they fulfil another permissive role. Secretion of mammalian leaderless proteins has also been described to be associated to membrane budding and to the release of vesicles into the medium [15,51]. However, ATP-dependency and a potential action of ABC-transporters in this process remain to be studied. From the current knowledge, it is difficult to relate these two processes that seem involved in secretion of mammalian leaderless proteins and to determine how ABC-transporters contribute to this release.

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