# Transport of Arachidonic Acid across the Neutrophil Plasma Membrane via a Protein-Facilitated Mechanism†

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ABSTRACT: Arachidonic acid is the rate-limiting substrate in the biosynthesis of leukotrienes in activated neutrophils. Liberation of arachidonate from intracellular membranes and uptake of exogenous arachidonate are the two principal mechanisms by which the cell can increase the level of this substrate. We investigated arachidonate uptake and export by using intact polymorphonuclear neutrophils and inside-out plasma membrane vesicles thereof. Here we show that the cellular uptake of arachidonate is energy dependent with an energy of activation ($E_A$) of 10.0 kcal/mol and half-saturated at an arachidonate concentration of 4.8 nmol/mg of cell protein. Protein-facilitated transport of arachidonate across the plasma membrane in either direction is sensitive to proteases, chemical protein modifying reagents, anion transport inhibitors, and, most notably, toward several structurally unrelated leukotriene $B_4$ receptor antagonists with $IC_{50}$ values in the range of 16−44 $\mu$M. The inhibitors did not inhibit the diffusional uptake of methyl arachidonate into neutrophils and inside-out plasma membrane vesicles, indicating that a transport protein is required for the rapid uptake of the free acid but not for the uptake of the ester. Other long-chain fatty acids did compete with the uptake of arachidonate in both assay systems, whereas leukotriene $B_4$ did not. This study documents a novel protein-facilitated transport mechanism for arachidonate in neutrophils, potentially involved in transcellular eicosanoid biosynthesis and $sPLA_2$-mediated arachidonate signaling in neutrophils.

Arachidonic acid (AA)[1] is the key precursor substrate for the biosynthesis of biologically potent eicosanoids such as prostaglandins (PG's), leukotrienes (LT's), and lipoxins. Since the limited intracellular availability of AA is a common bottleneck in the biosynthesis of these compounds (*1*), it is of great interest to unravel the mechanisms that provide unesterified AA for intracellular metabolism in order to define them as potential drug targets.

There are two principal mechanisms through which AA can be mobilized for intracellular eicosanoid synthesis: (i) intracellular release of AA and (ii) uptake of exogenous AA (*2*). Several phospholipases ($PLA_2$) that release AA from membrane phospholipids either intra- or extracellularly have been implicated in the biosynthesis of eicosanoids. Thus, several extracellular group II and V secreted $PLA_2$ ($sPLA_2$) forms (*3*) as well as a cytosolic group IV $PLA_2$ ($cPLA_2$) (*4*−*6*) and a $Ca^{2+}$-independent $PLA_2$ ($iPLA_2$) (*7, 8*) have attracted particular attention (*9*). Recent results obtained from mice deficient in $cPLA_2$ indicate that $cPLA_2$ plays a nonredundant role in allergic responses, post-ischaemic brain injury, and reproductive physiology (*10, 11*) and is critically involved in the biosynthesis of $PGE_2$, $LTB_4$, and $LTC_4$, at least in macrophages (*11*). However, in many other cell types, the situation is less well-defined, and both $cPLA_2$ and different $sPLA_2$ forms may be, either in parallel or sequentially, involved in AA release and eicosanoid generation (*12*−*17*).

At present, it seems very likely that one or several forms of $sPLA_2$ play an important role in diverse inflammatory processes, and although the mechanism is not completely understood, the extracellular release of arachidonate is generally regarded as one of the key events involved (*3, 18*). AA that has been released from one cell type can then be taken up by the same or another cell type and may be utilized for eicosanoid formation. In fact, transfer of [$^3$H]AA from labeled platelets to unlabeled neutrophils for [$^3$H]$LTB_4$ synthesis was the first example (*19*) of this type of biosynthetic pathway, now well recognized as "transcellular metabolism" (*20*−*23*).

Lipophilic compounds such as fatty acids, leukotrienes, lipoxins, and prostaglandins were long thought to enter and leave cells by simple diffusion. This is certainly not the case for prostaglandins (*24, 25*) and cysteinyl leukotrienes (*26, 27*), for which the respective carriers have recently been cloned. In addition, specialized transport systems in the neutrophil membrane which are involved in the uptake of lipoxins (*28*) and the export of $LTB_4$ (*29*) have been characterized.

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[1] Abbreviations: PG, prostaglandin; LT, leukotriene; PMN, polymorphonuclear leukocyte(s); $cPLA_2$, cytosolic phospholipase $A_2$; $sPLA_2$, secreted phospholipase $A_2$; AA, arachidonic acid; MeAA, methyl arachidonate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DTT, 1,4-dithiothreitol; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; NEM, *N*-ethylmaleimide; PCP, pentachlorophenol; CMPS, *p*-chloromercuriphenylsulfonic acid; MMTS, methyl methanethiosulfonate; CHC, α-cyano-4-hydroxycinnamic acid; DISA, 3,5-diiodosalicylic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

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With respect to transport of fatty acids in general, interest has been focused predominantly on the entry of long-chain fatty acids into parenchymal cells for consumption or storage (e.g., heart, liver, skeletal muscle, and adipose tissue) (*30*, *31*), and to date, five different putative fatty acid transport proteins have been identified (*32*). Selective antibody inhibition, liposome reconstitution, and functional genetic expression have successfully been employed to demonstrate the physiological role for at least two of them (*33*, *34*). However, these proteins differ in tissue occurrence. For example, all of them are expressed in adipose tissue and muscle which have a high metabolic need for fatty acids. Yet, the possible involvement of transport proteins in inflammatory cells that can quickly shuttle comparable small amounts of arachidonic acid in either direction of the membrane remains to be established.

Despite the advances that have been made to identify the eucaryotic fatty acid transport protein(s), the concept of protein-facilitated fatty acid transport is still highly controversial (*35*). Membrane translocation of fatty acids has also been interpreted by physicochemical models in which fatty acids partition between an extracellular albumin-bound pool, an aqueous phase, a membrane lipid phase, and intracellular binding sites. Rapid intracellular metabolism has been postulated as the driving force for fatty acid uptake. This model, suggesting that fatty acid uptake occurs predominantly by nonfacilitated diffusion, has been supported by uptake studies using isolated parenchymal cells and fatty acid translocation studies across artificial (protein-free) phospholipid membranes. The latter studies indicated an extremely fast movement across the phospholipid bilayer ($t_{1/2} = 20$ ms) (*36*, *37*). Thus, a protein-free phospholipid bilayer is no permeability barrier for fatty acids, suggesting that there is no universal requirement for a membrane protein to enhance the rate of fatty acid movement across membrane bilayers. However, these studies using artificial membranes do not answer the question whether fatty acids would spontaneously distribute into and dissociate from biological membranes of a specific cell type at a sufficient rate to accommodate biological needs.

In the present work, uptake and export mechanisms of AA have been studied using intact pig polymorphonuclear neutrophils and inside-out plasma membrane vesicles thereof. We demonstrate that AA transport across the neutrophil membrane in both directions is facilitated by one or more saturable membrane protein(s) that are sensitive to proteases, chemical protein modifying reagents, anion transport inhibitors, and a variety of structurally unrelated $LTB_4$ receptor antagonists. Hence, our results characterize a novel protein-facilitated transport mechanism for arachidonic acid across the neutrophil plasma membrane.

## MATERIALS AND METHODS

*Biochemicals.* Mineral oil (M-5904), lipopolysaccharide 026:B6 (L-3755), phenylmethylsulfonyl fluoride, Triton X-100, and PIPES dipotassium salt were from Sigma. Ficoll-Paque, Dextran T-500, and Con A Sepharose were obtained from Pharmacia LKB Biotechnology Inc. Arachidonic acid was from Fluka, and [5,6,8,9,11,12,14,15-$^3$H]arachidonic acid (230.5 Ci/mmol) was purchased from DuPont De Nemours. Tritiated methyl arachidonate was obtained by methylating [$^3$H]arachidonic acid with ethereal diazomethane/1% ethanol. Fatty acid free bovine serum albumin was from Boehringer Mannheim.

*Pig Polymorphonuclear Leukocytes (PMN).* Arterial blood was collected from 3−4 month old pigs using sterile $Na_2$-EDTA (2.14 g/L) as an anticoagulant. PMN were prepared by dextran sedimentation, hypotonic lysis of the remaining erythrocytes, and centrifugation on Ficoll-Paque, as described previously (*21*). PMN viability was determined by trypan blue exclusion and exceeded 95%.

*Preparation of Inside-Out Plasma Membrane Vesicles (Phagocytic Vesicles).* PMN were incubated with albumin-coated mineral oil droplets in the presence of 0.9 mM $CaCl_2$, 1.27 mM $MgCl_2$, and 10 ng/mL serum-treated lipopolysaccharide for 30 min at 37 °C (*38*). The resulting phagocytic vesicles within the PMN were liberated by nitrogen cavitation (200 psi for 20 min) and purified by Con A chromatography as described previously (*38*). Protein was determined using the bicinchoninic acid (BCA) assay (*39*). Electron microscopy revealed that the vesicle preparation consisted primarily of membrane-bound vesicles (0.1−1.2 $\mu$m) enclosing the phagocytized mineral oil droplet and some associated granules (*38*).

*Arachidonic Acid Uptake into Inside-Out Plasma Membrane Vesicles and PMN.* Purified PMN ($1.5 \times 10^6$ cells, 20 $\mu$g of protein, viability >95%) or plasma membrane vesicles (20 $\mu$g of protein) were incubated with 4.3 pmol of [$^3$H]AA (added as an 50% ethanolic solution) in 200 $\mu$L of modified relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES-HCl, pH 6.8) at 37 °C. The final ethanol concentrations never exceeded 0.5% and did not influence transport activity. Time-dependent internalization of [$^3$H]AA into cells or vesicles was terminated by the addition of 800 $\mu$L of ice-cold stop solution (0.5 mM pentachlorophenol/0.5% albumin in modified relaxation buffer). After a 10 min incubation period on ice, the sample was pipetted onto a Schleicher & Schuell filter, GF 52 (24 mm), and filtered using a filtration manifold (DN 025/1, Schleicher & Schuell, Germany). Subsequently, vesicles or cells on the filter were washed with 1 mL of stop solution and 3 mL of relaxation buffer. The filter was placed in a scintillation vial and left overnight in 5 mL of Rotizint Eco Plus (Roth, Germany) before radioactivity (internalized [$^3$H]-AA) was counted.

In addition, the total vesicle- or cell-associated [$^3$H]-arachidonate (AA in the outer leaflet of the membrane plus internalized AA) was determined by rapid filtration of the samples followed by a brief wash with 5 mL of modified relaxation buffer, and scintillation counting of the filter as described above.

*Determination of Initial Influx Rates.* Cumulative uptake of [$^3$H]AA into vesicles or PMN was determined at 10, 20, 30, and 40 s after addition of [$^3$H]AA as described above. The non-BSA-extractable [$^3$H]AA portion increased in a linear fashion during the first 40 s (Figure 1) and reflected the time-dependent uptake of AA. Initial influx rates were calculated from the slope of the linear portion of the curve. Since the initial influx rates are dependent on the membrane [$^3$H]AA concentration which determines the transport rate (see Results, Figure 1), it was necessary in some instances to normalize initial influx rates with respect to the [$^3$H]AA membrane concentration (determined as described above) in

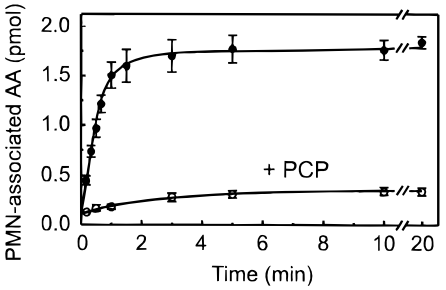


FIGURE 1: Uptake of arachidonate by intact PMN. Tritiated arachidonate (4.3 pmol) was incubated with $1.5 \times 10^6$ PMN/200 $\mu$L (20 $\mu$g of cell protein) in the absence (●) or presence (○) of 500 $\mu$M PCP. Uptake of [$^3$H]AA was determined after removal of externally bound radioactivity by the BSA/PCP washing procedure as detailed under Materials and Methods. Results are from three experiments (mean ± SD).

order to compare the effect of inhibitors on the translocation process alone.

*Validation of Methods.* Previous studies using intact cells as well as inside-out plasma membrane vesicles have established that fatty acids readily dissolve into the outer leaflet of the cell or vesicle membrane from where they are translocated to the inner leaflet by a time-dependent process. The use of a cold 200 $\mu$M phloretin/0.1% BSA or a 500 $\mu$M PCP/0.1% BSA stop solution efficiently removed surface-bound [$^3$H]fatty acid while blocking efflux of ligand already internalized into cells or vesicles, thereby permitting accurate quantitation of cumulative fatty acid uptake (*30, 32, 38, 40*−*43*). The cumulative $^3$H-uptake curve over the initial 10−50 s incubation reflected almost exclusively cellular/vesicular influx, and the slope of this portion of the curve provided an accurate quantitative measure of influx velocity. The studies below further characterize the nature of the arachidonate translocation process into inside-out plasma membrane vesicles and intact PMN.

## RESULTS

*Uptake of AA by Intact PMN.* Incubation of 4.3 pmol of [$^3$H]AA with $1.5 \times 10^6$ PMN (20 $\mu$g of protein/200 $\mu$L) yielded a time-dependent influx of AA into PMN. The amount of internalized AA increased linearly over the first 40 s, and then decreased progressively. From the slope of the cumulative uptake curve, an initial influx rate of 77.5 pmol min$^{-1}$ mg$^{-1}$ could be calculated; see Materials and Methods for details. In the presence of the AA transport inhibitor PCP (500 $\mu$M) (*38*), internalization of AA could be inhibited almost completely. A small portion of the applied AA (4.7% of total AA in the assay) was found associated with the cells that could not be extracted by the BSA washing procedure. This portion did not increase significantly during prolonged incubation times in the presence of PCP and was identical to the portion of non-BSA-extractable AA at zero time in incubations without PCP (Figure 1). BSA-resistant AA binding at zero time could not be saturated by AA (data not shown), and apparently reflected unspecific binding of AA to the membranes.

*Initial Uptake of [$^3$H]AA as a Function of the Concentration of AA Dissolved in the Outer Membrane Leaflet.* Arachidonate (4.3 pmol) was added to different concentrations of cells or inside-out vesicles (range: 5−60 $\mu$g of protein/200 $\mu$L) in albumin-free modified relaxation buffer.

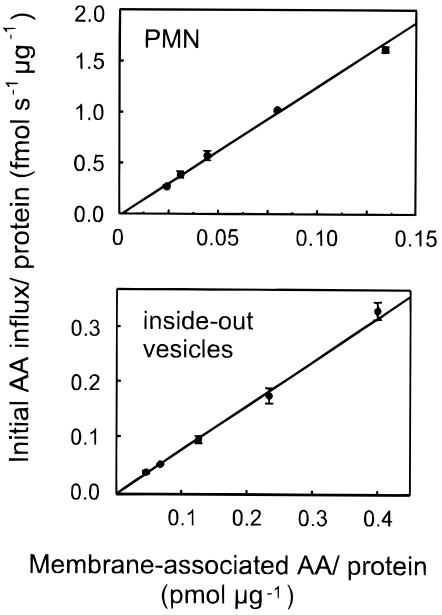


FIGURE 2: Dependence of the AA uptake velocity on the AA membrane concentration. Tritiated arachidonate (4.3 pmol) was incubated with intact PMN or inside-out plasma membrane vesicles (5−60 $\mu$g of protein) in 200 $\mu$L of modified relaxation buffer at 37 °C, and internalized [$^3$H]AA was determined at four time points 10−40 s after addition of radioactivity as described under Materials and Methods. Initial influx into a fraction of the cells or vesicles (corresponding to 1 $\mu$g of protein) was calculated and plotted against the concentration of unesterified AA dissolved in the membrane of the cells or vesicles (1 $\mu$g of protein). A linear dependency of the initial influx velocity on the membrane-associated [$^3$H]AA was obtained. Experiments were performed in triplicate (mean ± SD).

Rapid filtration and washing of cells or vesicles with albumin-free buffer revealed that a constant fraction of the total applied [$^3$H]AA associated with the cells or vesicles (2.8 and 1.9 pmol of AA, respectively) and that this portion (AA in the outer membrane leaflet plus internalized AA) did not change during the duration of the experiment. Since the concentration of cells and vesicles in the experiments was varied, different concentrations of unesterified AA dissolved in the membrane (24−400 fmol of AA/$\mu$g of protein at zero time) were obtained.

When the initial AA influx into cells or vesicles (1 $\mu$g of protein) was calculated and plotted against the actual concentration of unesterified AA in the cell or vesicle membrane (1 $\mu$g of protein), a linear relationship was observed (Figure 2). Thus, the influx velocity of AA into cells or vesicles appeared to be linearly dependent on the concentration of arachidonate that is dissolved in the outer membrane leaflet, but not on the unbound AA concentration in the water phase which remained constant during the experiments.

*Saturation Kinetics of Arachidonate Uptake into PMN.* To investigate if the AA uptake into PMN is saturable, we monitored the rate of [$^3$H]AA influx as a function of the concentration of AA dissolved in the outer plasma membrane leaflet at the beginning of the experiment. Addition of [$^3$H]-AA (1.3 nM−5 $\mu$M) to $1.5 \times 10^6$ PMN (20 $\mu$g of protein/200 $\mu$L) yielded a membrane concentration between 0.24 and 264 pmol of AA/20 $\mu$g of PMN protein. The initial influx rates appeared to level off as the arachidonic acid concentration in the external membrane leaflet increased. The data were fitted against the Michaelis−Menten equation by

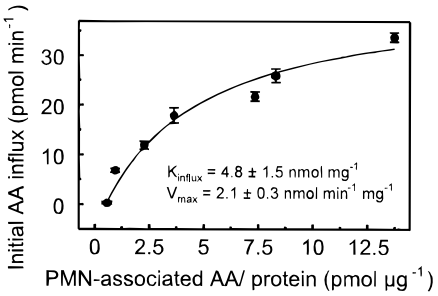


FIGURE 3: Saturation kinetics of AA influx into PMN. Experiments were carried out in the presence of a constant amount of [$^3$H]AA. Increasing quantities of unlabeled AA were added to $1.5 \times 10^6$ PMN/200 $\mu$L (20 $\mu$g of cell protein) to achieve a concentration of unesterified AA in the membrane of 0.012−13.2 pmol/$\mu$g. Uptake was measured at four time points between 10 and 40 s, and initial influx velocities were calculated as described under Materials and Methods. A half-maximal influx rate, $K_{influx}$, was calculated for an arachidonate concentration of 4.8 ± 1.5 nmol/mg of cell protein, and a maximal influx rate of 2.1 ± 0.3 nmol min$^{-1}$ mg$^{-1}$) was obtained. Results are from three experiments (mean ± SD).

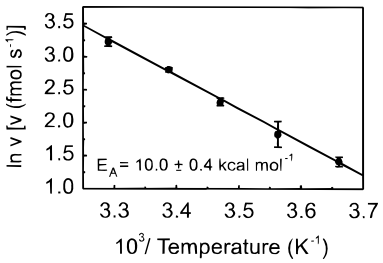


FIGURE 4: Arrhenius plot of [$^3$H]AA influx into neutrophils. Uptake of [$^3$H]AA was measured at four different points along the time course (10−40 s), and influx rates (0−30 °C) were determined as described under Materials and Methods. This graph indicates a strictly linear relationship with no apparent breaks in the curve. The slope signifies an activation energy of 10.0 ± 0.35 kcal/mol. Results are from three experiments (mean ± SD).

nonlinear regression analysis (Figure 3). A half-maximal influx rate, $K_{influx}$, was calculated for an arachidonate concentration of 4.8 ± 1.5 nmol/mg of cell protein, and a maximal influx velocity of 2.1 ± 0.3 nmol min$^{-1}$ mg$^{-1}$ was determined.

*Protease Sensitivity.* The possible involvement of membrane proteins in the uptake mechanism can be probed by determining the effect of proteases on the uptake velocity. Previously, it had been demonstrated that protease treatment of inside-out vesicles for 12 h at 37 °C led to an almost complete loss of transport activity, suggesting that partially exposed membrane carrier proteins are involved in the export of AA in PMN. Proteolytic digestion of intact PMN by trypsin and Pronase for 4 h at 37 °C resulted in 41 ± 7% and 38 ± 4% loss, respectively, of transport activity relative to control cells that were incubated simultaneously without addition of proteases. Only partial inactivation was seen which may be due to steric protection of the putative carrier by the membrane during the relatively short incubation time.

*Temperature Dependence of Arachidonate into PMN.* To assess the temperature dependence of the transport reaction, we also determined the rate of arachidonate uptake into PMN as a function of temperature between 0 and 37 °C. Transport activity increased dramatically between 0 and 30 °C and to a lesser extent between 30 and 37 °C. The data (0−30 °C) are graphed in Figure 4 in the form of a conventional Arrhenius plot. The results convey a linear relationship exhibiting a rather shallow slope that translates into an activation energy of 10.0 ± 0.4 kcal/mol which is close to the activation energy of 11.6 ± 0.3 kcal/mol previously determined for AA influx into inside-out vesicles (*38*).

*Inhibition of AA Uptake into Inside-Out Vesicles and Intact PMN.* Inhibition of arachidonate transport by inhibitors is another criterion to define transport proteins and may even allow differentiation between different anion transport processes. Some anion transport inhibitors such as pentachlorophenol (PCP), phloretin, and diiodosalicylic acid (DISA) showed a strong inhibition of initial AA influx (99.8−90% inhibition) into inside-out vesicles and intact PMN when tested at an inhibitor concentration of 400−1000 $\mu$M. Several other common anion transport inhibitors such as niflumic acid, α-cyano-4-hydroxycinnamic acid (CHC), probenicid, and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) were less potent or inactive. Notably, the inhibitors did not inhibit the association of arachidonic acid with the membranes (data not shown) but efficiently prevented the internalization of AA. For PCP, a half-maximal influx velocity into PMN and inside-out vesicles was determined at PCP concentrations of 60 ± 4 and 2.6 ± 0.4 $\mu$M (mean ± SD), respectively.

*Inhibition of Arachidonate Uptake by Protein Modifying Reagents.* Transport proteins are very often sensitive to chemical modification reagents that are designed to react with different types of amino acids in proteins with a reasonable specificity. Three different thiol reagents were preincubated with PMN or vesicles at 0 °C for 45 min. After warming-up to 37 °C for 15 min, the transport assay was started by addition of AA as described above. At a concentration of 0.5−1 mM, *p*-chloromercuriphenylsulfonic acid (CMPS), *N*-ethylmaleimide (NEM), and methyl methanethiosulfonate (MMTS) inhibited the uptake of arachidonate into intact PMN almost completely (>98% inhibition of AA uptake) without affecting the association of AA with PMN membranes. Interestingly, AA influx into inside-out membrane vesicle was not or only slightly inhibited by these reagents (Table 1).

After preincubation of vesicles or cells with 10 mM phenylglyoxal, a lipophilic arginyl reagent, both uptake processes were inhibited by 70% and 97%, respectively. Butanedione, which is a less hydrophobic arginyl reagent, was a weaker inhibitor at the same concentration. A tyrosyl modifying reagent, *N*-acetylimidazole, displayed an inhibitory effect (68% of untreated control) on AA influx into PMN but did not inhibit the uptake of AA into the inside-out vesicles.

*Effect of $LTB_4$ Receptor Antagonists on AA Uptake.* Most of the developed $LTB_4$ antagonists exhibit a high affinity (low nanomolar range) for the $LTB_4$ receptor (*44*). At higher concentrations, at least some $LTB_4$ receptor antagonists such as SC-41930 also exert inhibitory effects on leukotriene metabolism that are not related to antagonism of $LTB_4$ receptors (*45*). We tested four different $LTB_4$ receptor antagonists for inhibitory activity in the AA transport assays. At a concentration of 100 $\mu$M, the selected receptor antagonists CP-105.695 (*46*), SC-41930 (*47*), SC-53228 (*47*), and LY-255283 (*48*) all completely blocked AA uptake into intact PMN as well as into the vesicles (Table 1). The membrane association of [$^3$H]AA as determined by rapid

Table 1: Inhibition of [$^3$H]AA Uptake into PMN and Vesicles[a]

| inhibitor | concn (mM) | initial AA influx velocity (%) PMN | inside-out vesicles |
|---|---|---|---|
| none | | 100 ± 10 | 100 ± 6 |
| anion transport inhibitors | | | |
| PCP | 0.5 | 2 ± 3 | 1.3 ± 0 |
| phloretin | 0.4 | 8 ± 2 | 9 ± 6 |
| DISA | 0.5 | 0.21 ± 0.2 | 10 ± 7 |
| niflumic acid | 0.5 | 27 ± 4 | 23 ± 2 |
| CHC | 0.4 | 68 ± 15 | 105 ± 2 |
| SITS | 0.5 | 46 ± 2 | 74 ± 3 |
| probenecid | 0.5 | 80 ± 7 | 137 ± 5 |
| protein-modifying reagents | | | |
| CMPS | 0.5 | 2 ± 2 | 74 ± 5 |
| NEM | 1 | 1 ± 3 | 78 ± 4 |
| MMTS | 1 | 1 ± 1 | 127 ± 15 |
| phenylglyoxal | 10 | 3 ± 2 | 30 ± 4 |
| butanedione | 10 | 81 ± 6 | 88 ± 8 |
| *N*-acetylimidazole | 10 | 32 ± 2 | 99 ± 4 |
| $LTB_4$ receptor antagonists | | | |
| SC-41930 | 0.1 | 0 ± 0.5 | 11.9 ± 7 |
| SC-53228 | 0.1 | 1 ± 0 | 13.4 ± 1 |
| LY-223982 | 0.1 | 6 ± 2 | 1 ± 0 |

[a] Values are means ± SE ($n = 3$). Initial influx rates were determined in the presence of the inhibitors; see Results for details.

filtration of the vesicle or cell suspension incubates was reduced by these compounds by 33−40%. To measure the effect on the transport step alone, initial influx rates were normalized with respect to the total membrane-associated arachidonate. The initial influx velocity of AA into cells and vesicles was determined as described under Materials and Methods, and the $IC_{50}$ values for the inhibitors were defined as the inhibitor concentration that reduces the initial influx velocity to 50%. Dose−response curves were run for each receptor antagonist in triplicate, and sigmoidal inhibition curves were obtained from which $IC_{50}$ values in the range of 16−44 $\mu$M for both transport processes (i.e., uptake into PMN and inside-out vesicles) were calculated (Figure 5).

*Methyl Arachidonate Is Taken Up into PMN and Inside-Out Plasma Membrane Vesicles by Free Diffusion.* Non-ionizable fatty acid ester derivatives are thought to be taken up into cells entirely by uncatalyzed diffusion with a velocity which is orders of magnitude higher than for the fatty acid anion (*49*). To test the specificity of AA transport inhibitors and proteases for the putative AA carrier, we investigated the uptake of arachidonic acid methyl ester (MeAA) by PMN and inside-out vesicles.

The influx rate of MeAA into vesicles was at least 25-fold faster than the corresponding influx of AA at pH 6.8. In fact, MeAA influx was too fast (37 °C: $t_{1/2} < 15$ s; 0 °C: $t_{1/2} = 40$ s) to be measured accurately with the conventional filtration assay. Internalization of MeAA into PMN occurred at an even higher rate, reaching maximal intracellular MeAA levels already after 15−30 s.

After incubation of inside-out vesicles (20 $\mu$g of protein) with 4.3 pmol of [$^3$H]MeAA for 10 min at 37 °C, 73% of the vesicle-associated radioactivity could not be extracted by PCP/0.1% BSA. After physical destruction of the vesicles by sonication, 85% of BSA-resistant vesicle-associated [$^3$H]-MeAA of intact vesicles could be extracted by 0.1% BSA treatment without affecting the recovery of the membranes. This indicates that MeAA has indeed been internalized and cannot be removed from the vesicle interior by the BSA

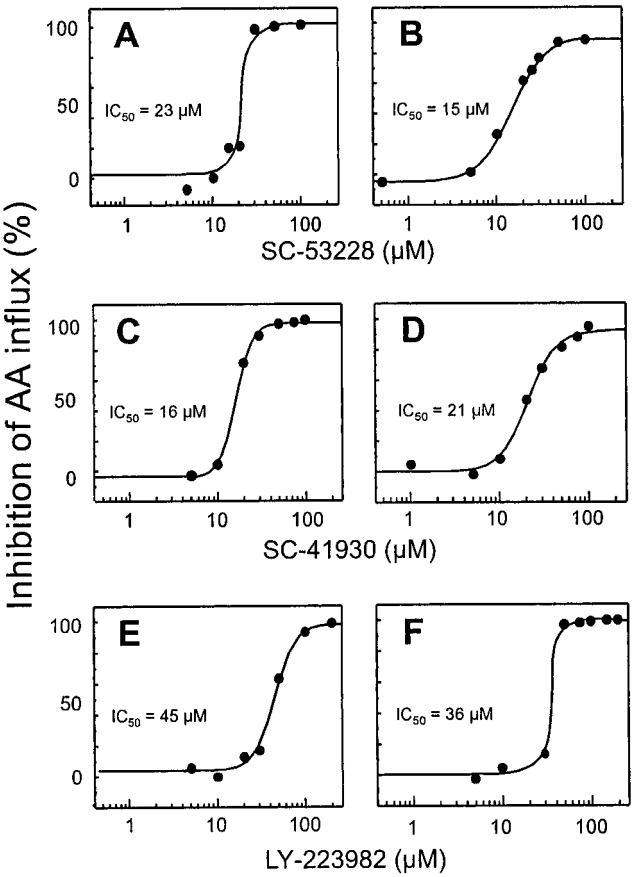


FIGURE 5: Dose dependencies of $LTB_4$ receptor antagonists with respect to inhibition of [$^3$H]AA internalization. Inhibitors were preincubated at the concentration stated for 45 min at 0 °C and for 15 min at 37 °C with $1.5 \times 10^6$ PMN (20 $\mu$g of cell protein) or inside-out vesicles (20 $\mu$g of protein), respectively. Incubations were started by addition of 4.3 pmol of [$^3$H]AA/200 $\mu$L. The $IC_{50}$'s were determined by measuring the inhibition of the initial influx rates (normalized with respect to the [$^3$H]AA membrane concentration) according to the procedures described under Materials and Methods. Results are from experiments performed in triplicate.

washing procedure unless vesicles were physically destroyed. Under similar conditions, 70% of the added MeAA was taken up by PMN (20 $\mu$g of protein). Pretreatment of intact PMN or vesicles with Pronase or trypsin under the conditions previously used to inhibit AA uptake (see above) did not have any effect on MeAA uptake.

A series of inhibitors of arachidonic acid transport (PCP, phloretin, DISA, and the $LTB_4$ receptor antagonists SC-41930 and LY-223982) were tested for their effect on the accumulation of [$^3$H]AA and [$^3$H]MeAA both in inside-out vesicles and in PMN (Figure 6). After a 30 min incubation period, external AA was removed by the BSA/PCP washing procedure; 44.8% of the applied [$^3$H]AA and 47.3% of the [$^3$H]MeAA were found PMN-associated (internalized plus unspecifically bound AA). In the case of the vesicles, 19.6% [$^3$H]AA and 49.6% [$^3$H]MeAA were found vesicle-associated after an incubation period of 30 min. Accumulation of internalized, non-BSA-extractable [$^3$H]AA and [$^3$H]MeAA in control cells and vesicles was set to 100% and compared with their accumulation in cells and vesicles in the presence of the inhibitors (Figure 6). None of the inhibitors had a significant effect on the accumulation of MeAA while AA uptake was partially or almost completely prevented by the inhibitors. PCP (0.5 mM) and the $LTB_4$ receptor antagonists

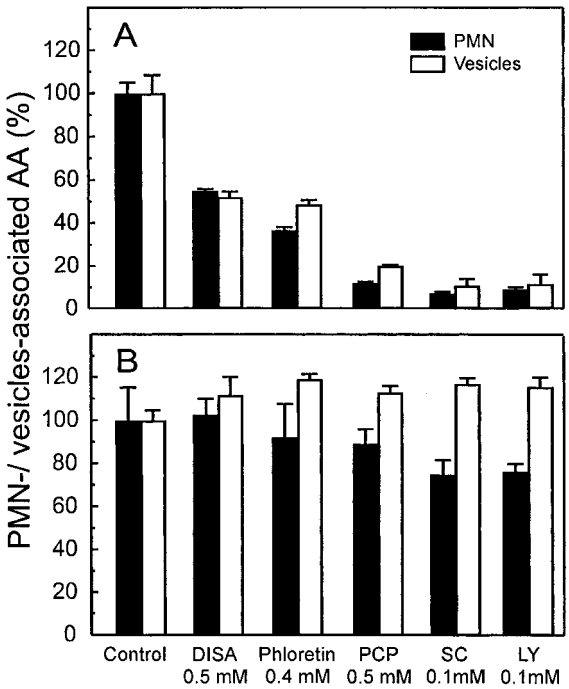


FIGURE 6: Inhibition of [$^3$H]AA and [$^3$H]MeAA uptake into PMN and inside-out plasma membrane vesicles. PMN and inside-out vesicles were incubated with 4.3 pmol of tritiated AA (A) and MeAA (B) for 30 min, respectively. At the end of the incubation time, cell- or vesicle-associated radioactivity was determined after removal of externally bound ligand by the BSA/PCP washing procedure as described under Materials and Methods. Bars indicate the percentage of radioactivity associated with cells or vesicles in the absence (control) or presence of the inhibitors DISA, phloretin, PCP, SC-41930 (SC), and LY-255283 (LY). Experiments were performed in triplicate (mean ± SD).

(100 $\mu$M) were especially effective in preventing the cellular or vesicular influx of AA (Figure 6).

The small fraction of AA found associated with the cells and vesicles in the presence of these inhibitors reflects in part unspecific but BSA-resistant binding of AA to membranes since extrapolation of the cumulative uptake curves (not shown) to zero time revealed fractions of 3.8 ± 3.5% and 0.6 ± 0.2% AA (in cells and vesicles, respectively) that bound almost instantaneously to the membranes and was not sensitive to PCP. Although phloretin and DISA inhibited the initial influx velocity of AA by more than 90% (Table 1), these inhibitors were less effective during the 30 min incubation time, yielding only partial prevention of AA accumulation.

*Competition of AA Uptake by Other Fatty Acids.* Fatty acid transport proteins generally display a rather broad substrate specificity toward long-chain fatty acids (*43*, *49*, *50*). We probed the putative AA transporters in the vesicles and intact PMN by competition of [$^3$H]AA transport with unlabeled linoleic, linolenic, and arachidonic acid as well as the hydroxylated AA metabolite, $LTB_4$. Transport assays were initiated by simultaneous addition of 20 nM tritiated arachidonic acid and 100 $\mu$M fatty acid or 10 $\mu$M $LTB_4$, respectively. Linoleic, linolenic acids, and arachidonic acid but not $LTB_4$ decreased the portion of [$^3$H]arachidonate dissolved in the outer leaflet of the membrane by 68, 30, and 36%, respectively. To determine the effect of the competing substrates on the translocation process alone, influx rates were normalized with respect to the membrane-bound [$^3$H]AA. Linoleic acid (100 $\mu$M) and arachidonic acid

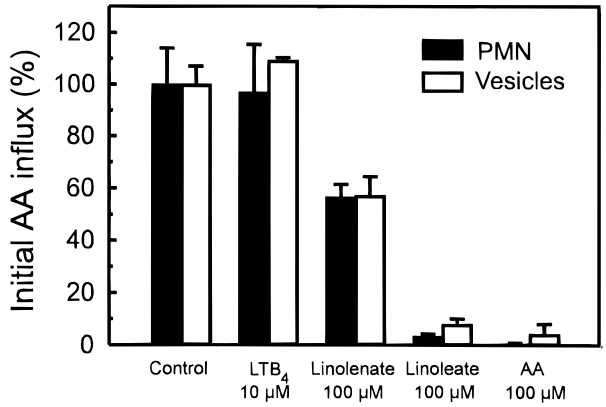


FIGURE 7: Inhibition of [$^3$H]AA uptake by fatty acids and $LTB_4$. Incubations were initiated by simultaneous addition of unlabeled lipid and [$^3$H]AA. Initial influx rates were determined as detailed under Materials and Methods. Initial influx rates of incubations with unlabeled lipid/[$^3$H]AA mixtures (normalized with respect to the [$^3$H]AA membrane concentration) were compared with those which received [$^3$H]AA alone (100%).

(100 $\mu$M) inhibited internalization of membrane-associated [$^3$H]AA by more than 90% while linolenic acid (100 $\mu$M) yielded only 67% inhibition (Figure 7). In contrast, $LTB_4$ at a receptor saturating concentration (10 $\mu$M) did not inhibit uptake of [$^3$H]AA into PMN and vesicles, demonstrating that $LTB_4$ receptors are not involved in the membrane translocation process of AA.

## DISCUSSION

Free fatty acids in plasma are largely complexed to albumin. Unbound fatty acids dissolve readily into the outer leaflet of cell membranes, thus leading to a situation in which unesterified fatty acids distribute between albumin complexes, water, and membranes. In the present investigation, we show that unesterified AA which is dissolved in the PMN membrane is rapidly internalized by the cells in a time-dependent (Figure 1) and saturable process (Figures 2 and 3) which is compatible with a carrier-mediated process. It could be argued that AA penetrates the cell by a non-rate-limiting diffusion process and that saturation reflects filling of intracellular binding sites or intracellular metabolism. Since PMN rapidly incorporate AA into membrane lipids, this possibility cannot be excluded. However, the low half-saturating AA concentration for uptake into PMN lies within the range that has previously been determined for other fatty acids and cell types (*30*, *43*, *49*, *50*). Furthermore, saturable transport kinetics for AA in the reverse direction have been demonstrated using inside-out plasma membrane vesicles from PMN (*38*). Inhibition of [$^3$H]AA uptake into PMN and inside-out vesicles by proteases, common anion transport inhibitors, chemical protein modification reagents, competing fatty acids, and $LTB_4$ receptor antagonists (Figure 8) lends further support to the hypothesis that AA transport across the neutrophil membrane is facilitated by membrane protein(s). The diffusional, unspecific uptake rate determined in the presence of the AA transport inhibitor PCP is less than 2% of the initial uptake rate in the absence of this inhibitor (Table 1). In contrast, uptake of methyl arachidonate appears to proceed entirely through an uncatalyzed diffusion process that could not be inhibited by any of the above-mentioned treatments. This finding suggests also that the unesterified carboxylate group of AA is required to be recognized as substrate by the AA transport protein(s).

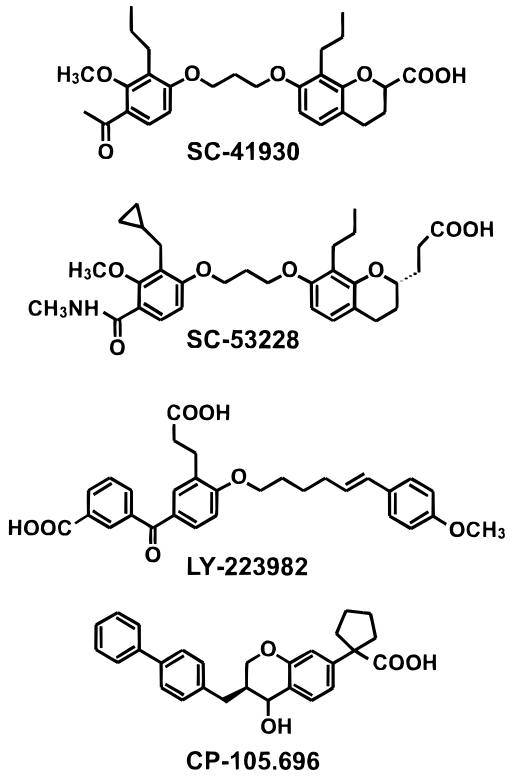


FIGURE 8: Structures of $LTB_4$ receptor antagonists that inhibit arachidonate transport.

Sensitivity of fatty acid transport toward a variety of transport inhibitors as demonstrated in this study has commonly been regarded as strong evidence for a protein-mediated transport process (*30*). However, these experiments have been criticized because it is necessary to demonstrate that the inhibitor would not prevent the insertion of fatty acid into the outer leaflet of the phospholipid bilayer, which would be the first critical step also in a diffusional uptake (*35*). As expected, the anion transport inhibitors as well as the protein-reactive reagents did not exert any appreciable effect on the membrane binding of [$^3$H]AA whereas the more lipophilic $LTB_4$ receptor antagonists as well as unlabeled fatty acids did compete with [$^3$H]AA binding to some extent. This obstacle could be overcome by comparing the influx rates in the presence or absence of the inhibitors after normalization of the influx rates with respect to the concentration of unesterified AA dissolved in the membrane. Thus, the effect of the inhibitors on the translocation step alone could be demonstrated (Table 1, Figures 5 and 7). It has also been argued that inhibitors may not inhibit what they are aimed for but rather block a potential enzymatic driving force, generating a favorable fatty acid gradient for the uptake of fatty acids, or may change the physical properties of the membrane barrier (*35*).

However, the absence of AA/MeAA metabolism in the vesicle system has been demonstrated (data not shown) and—with the exception of the thiol reagents and the tyrosyl reagent *N*-acetylimidazole—all of the inhibitors (Table 1) which show a strong inhibition in the vesicle system (used to study AA export) also proved to be strong inhibitors of cellular uptake and vice versa. The selective inhibition of AA uptake into PMN by thiol reagents and *N*-acetylimidazole could be explained by the different sensitivity of two AA carriers involved in export and uptake of AA, but may also be due to inhibition of protein(s) that promote(s) intracellular arachidonate metabolism in the PMN assay. Both AA esterification and MeAA transesterification into intracellular membrane phospholipids occurred rapidly after internalization of the lipids into PMN. However, no metabolites of AA or MeAA could be detected in the cell supernatant after the incubation, indicating that the extracellular substrates remained intact in these experiments (data not shown).

In addition, diffusional uptake of MeAA is not affected by any of the inhibitors used (Figure 6). Thus, it is unlikely that the inhibitors exert a major impact on the physical membrane barrier properties toward lipids in general.

Interestingly, the inhibition profile by transport inhibitors and fatty acids and also the $IC_{50}$ values of the $LTB_4$ receptor antagonists for AA transport in both directions appear to be surprisingly similar (Figure 6). Together with the very similar activation energy for export [11.6 kcal/mol, (*38*)] and import (10.0 kcal/mol, Figure 3), this suggests that a common protein may be involved in AA export and uptake such as flippase-type carriers that catalyze the flip-flop of amphipathic ions (*51*, *52*) from one leaflet of the membrane to the opposite leaflet in either one or both directions. This transport system may accept other long-chain fatty acids but not $LTB_4$ with high affinity as assessed by substrate competition experiments (Figure 7). However, it will be necessary to purify and reconstitute the putative arachidonate transport protein(s) from neutrophils to study the underlying transport mechanism in more detail.

The results presented here indicate a novel carrier-mediated transport system in PMN that serves to promote the uptake of exogenous AA which may be of considerable physiological relevance in inflammatory conditions. Thus, exogenous arachidonate, which would otherwise be trapped in plasma by high-affinity binding sites of albumin (*53*), may become available for PMN through the activity of a high-affinity arachidonate carrier. When stimulated by calcium ionophores, neutrophils release substantial quantities of free arachidonic acid and synthesize $LTA_4$, $LTB_4$, and 5-hydroxyeicosatetraenoic acid. Paradoxically, many receptor-mediated agonists are, at best, weak stimuli of AA release by $PLA_2$'s. In fact, leukotriene biosynthesis in PMN stimulated with a variety of natural agonists such as the C3b component of complement (serum-treated zymosan), the Fc portion of IgG (heat-aggregated IgG), and fMet-Leu-Phe (agonists at the C3b, Fc, and fMLP receptors, respectively) is of low magnitude and is often not detectable by HPLC procedures. However, $LTB_4$ synthesis can be dramatically increased when PMN are stimulated in the presence of exogenous AA, suggesting that the enzymatic machinery is limited by the supply of intracellular arachidonic acid (*23*, *54*). On the other hand, elegant experiments by Borgeat and co-workers have recently indicated that neutrophil-derived adenosine may down-regulate the biosynthesis of leukotrienes in PMN stimulated with natural proinflammatory agonists. Adenosine deaminase or adenosine receptor antagonists neutralized the effect of adenosine and led to significant leukotriene formation (*55*, *56*).

With respect to the complex in vivo situation, a somewhat unexpected class of arachidonate transport inhibitors, the $LTB_4$ receptor antagonists, may be useful pharmacological tools to investigate the relevance of transcellular arachidonate pathways and uptake of exogenous AA for eicosanoid biosynthesis.

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