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Experimental and Theoretical Investigation of Arachidonic Acid Uptake in Macrophages

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Abstract—The kinetics of ^{3}H-labeled arachidonic acid (AA, 10^{-10}-10^{-5} M) incorporation into murine peritoneal macrophages was investigated. During the incorporation of AA into the cells, the steady state was reached at 10 h. The level of incorporation consisted of 48-50% for nanomolar concentrations and 28-30% for micromolar concentrations of AA. Exogenous AA in micromolar but not nanomolar concentrations stimulated [^{3}H]AA release from intracellular stores of pre-labeled cells. A mathematical model fitting the behavior of the experimental system is proposed. The difference in the level of uptake of AA in nanomolar and micromolar concentrations is explained by the activation of AA release from intracellular stores at high concentrations of exogenous AA.

Key words: arachidonic acid, macrophages, metabolism, kinetics, modeling

Arachidonic acid (AA) serves as a substrate for the biosynthesis of a variety of physiologically active eicosanoids. They include prostanoids, leukotrienes, and lipoxins. These substances display diverse effects on bodily functions *in vivo* and on cellular responses *in vitro* [1]. Moreover, AA itself can directly affect several functions of the whole organism and of cells [2, 3]. The processes of AA transport, synthesis, and metabolism are currently under intensive investigation. It is known that under normal physiological conditions the content of free AA does not exceed nanomolar concentrations. The bulk of AA is stored in the phospholipids of cell membranes and lipoproteins [4]. However, during the inflammation the concentration of free AA raises up to several tens of micromolar [4].

The current commonly accepted view of the limitation of eicosanoid biosynthesis is the availability of free intracellular AA [5]. There are two possibilities for an increase in free AA concentration within a cell: the release of endogenous AA from phospholipids of cellular membranes, and the entrance of exogenous AA from the extracellular space [4]. Recently, a difference in the metabolism of endogenous and exogenous AA has been shown [6-8]. Within cells, AA is usually released by different phospholipases of the A_2 (PLA_2) family [9-11]. In macrophages, biosynthesis of prostanoids and leukotrienes mainly depends on cytosolic PLA_2 activity [12]. In other cells this process is regulated by both cytosolic and secretory PLA_2 [13-15]. Secretory PLA_2 also plays an important role in AA release into the extracellular space [9, 16]. This AA can be incorporated into cells of its or another type and can be used for the synthesis of eicosanoids [17, 18]. Thus, the processes of AA release and incorporation affect signal transduction between cells and tissues. The entrance of AA into cells from the extracellular medium comprises absorption on the cell plasma membrane, moving across phospholipid bilayer, and dissociation into the cytosol. Then AA is bound into diverse phospholipids [19]. The mechanism of this process is still under active discussion. The main discrepancies concern the mechanism of fatty acid (FA) transmembrane movement. Based on physicochemical properties of FAs and membranes, some investigators assume that FAs enter cells by simple diffusion [19-21]. Others suggest that FAs are transported by FA-binding proteins of the plasma membrane [22-26]. It should be noted that both mechanisms of FA transport are very fast and proceed within the second range time, whereas AA metabolism and eicosanoid biosynthesis take significantly longer (minutes, hours).

We showed previously that the kinetics of prostanoid synthesis by macrophages depends on the source of AA supplying the polyenzymatic system [6, 7]. Thus, in the current work we performed a detailed investigation of AA incorporation kinetics using a wide range of AA concentrations and the kinetics of [^{3}H]AA release from equilib-

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rium-labeled murine peritoneal macrophages stimulated by different AA concentrations. A mathematical model fitting the behavior of the experimental system over a wide range of concentration is proposed.

MATERIALS AND METHODS

[^{3}H]AA was purchased from Amersham (USA), RPMI-1640 culture medium from ICN (USA), and fetal calf serum (FCS) from Sigma (USA). All other reagents were of analytical grade.

Murine peritoneal macrophages were obtained from F1 male mice (18-20 g weight). The macrophages were isolated in RPMI-1640. Then FCS was added to the final concentration of 7.5%. The cells ($1\cdot10^6$/ml) were incubated in 96-well plates (Nunc, Denmark) in an atmosphere with 5% CO_2 at 37°C. After 2 h, the cells were washed twice with saline phosphate buffer and used for further work.

To investigate the kinetics of AA incorporation, fresh RPMI-1640 with different concentrations of AA but the same concentration of [^{3}H]AA (10^{-10} M, $1.92\cdot10^{-8}$ Ci/ml) was added to the cells. Aliquots (180 μl) of culture medium were taken at various times. Radioactivity in the samples was measured by liquid scintillation counting.

To study AA release, the cells were labeled with [^{3}H]AA (10^{-10} M, $1.92\cdot10^{-8}$ Ci/ml) for 22-24 h in RPMI-1640 with 2% FCS. The labeled cells were washed twice with phosphate saline buffer. Then fresh medium with different concentration of "cold" AA or 5 μM A23187 was added. Aliquots (180 μl) of culture medium were taken at various times. Radioactivity in the samples was measured by liquid scintillation counting.

The mathematical model was computed using a program written in Transform language, which is a part of the program Sigma Plot for Windows v1.0.

RESULTS AND DISCUSSION

Uptake of AA by macrophages. To study the kinetics of AA uptake by murine peritoneal macrophages, fresh media containing 10^{-10}-10^{-5} M AA with concentration step of one order of magnitude were added to the cells. All the media contained the same quantity of [^{3}H]AA. The radioactivity in the cell culture media was measured as a function of time (Fig. 1). The results showed that the concentration range of AA can be divided into two intervals. The first includes concentrations 10^{-10}-10^{-7} M. The experimental curves were identical over this interval; therefore, the results only for 10^{-10} and 10^{-7} M are presented in Fig. 1. These concentrations of AA are common for normal physiological processes *in vivo* [4]. The next interval includes micromolar concentrations of the substance, these being typical for inflammatory processes [4]. The amount of incorporated AA linearly depends on the concentration of added AA in the intervals 10^{-10}-10^{-7} and 10^{-6}-10^{-5} M. Thus, the relative AA incorporated into the cells is the same for each of these intervals (Fig. 1a).

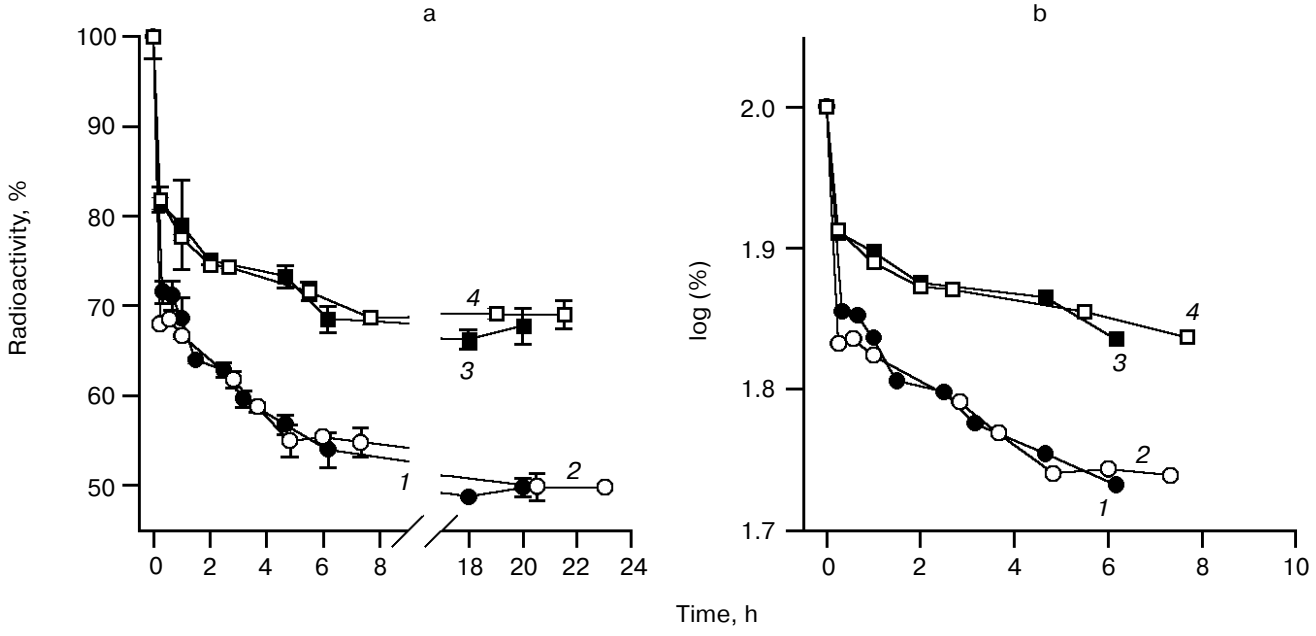

Fig. 1. Kinetics of [^{3}H]AA incorporation into murine peritoneal macrophages: a) radioactive label in media (% of initial content); b) data from (a) in semi-logarithmic coordinates. Concentration of applied AA (M): *1*) 10^{-10}; *2*) 10^{-7}; *3*) 10^{-6}; *4*) 10^{-5}. Conditions: AA (10^{-10}-10^{-5} M) contained the same amount of radioactivity, which was taken as 100% in the initial time.

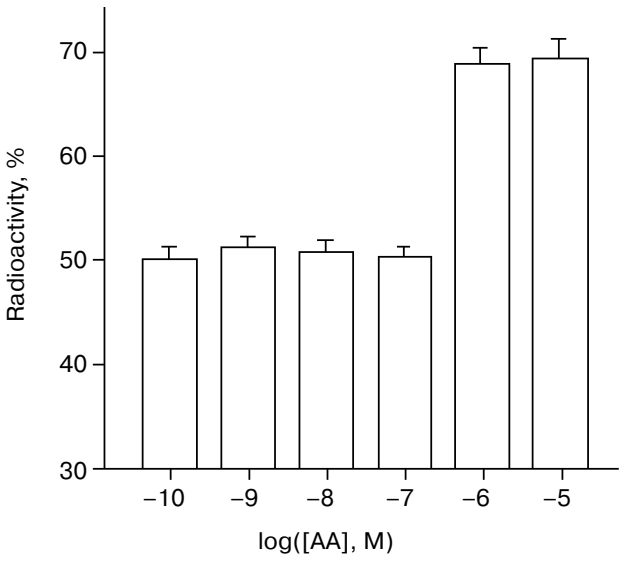

Fig. 2. Level of radioactivity in the cell culture medium after 20-h incubation with different concentrations of AA. Conditions as described in legend of Fig. 1.

By plotting the experimental results in semi-logarithmic coordinates, two areas can be noted on the curves. This means that at least two processes with remarkable difference in the rates are involved in AA uptake (Fig. 1b).

For the 10^{-10}-10^{-7} and 10^{-6}-10^{-5} M ranges of AA concentrations, 50-53% and 70-72% of the radioactivity remained in the medium after 20 h, respectively (Fig. 2).

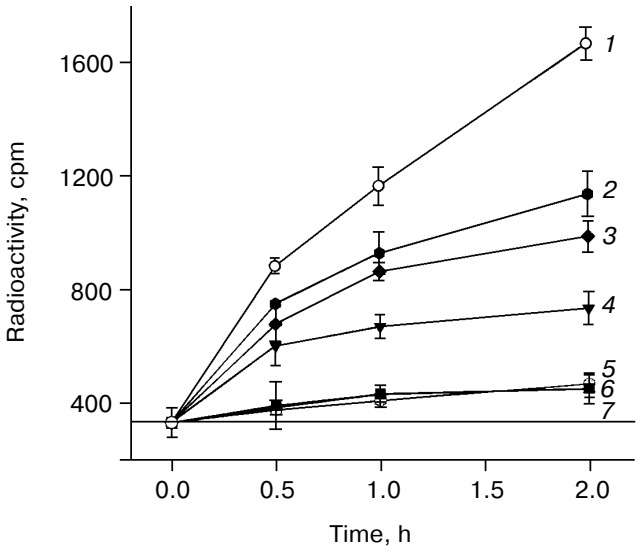

Fig. 3. Kinetics of radioactivity release from murine peritoneal macrophages stimulated by different agents. Concentration of A23187, 5 μM (*1*); AA (M): 10^{-5} (*2*); $10^{-5.5}$ (*3*); 10^{-6} (*4*); 10^{-7} (*5*); 10^{-8} (*6*); unstimulated release (control) (*7*). Conditions: cells were labeled with [^{3}H]AA for 24 h and washed three times with saline phosphate buffer. The stimuli were added after 1 h.

These results suggest that additional pathways of AA metabolism are activated by inflammatory AA concentrations.

AA release from intracellular stores of macrophages. The main pathway of AA release from cells is hydrolysis of phospholipids catalyzed by phospholipase A_2 [9-11]. Thus, the processes of AA release can be evaluated as radioactivity released to the medium from [^{3}H]AA-labeled cells. To investigate the reasons for differences in the kinetics of incorporation of AA applied to the cells in normal and inflammatory doses, the influence of exogenous AA on AA release from intracellular stores was studied. "Cold" AA at concentrations 10^{-8}-10^{-5} M was added to the [^{3}H]AA pre-labeled cells. Then the radioactivity in the medium was determined. We found that AA in normal physiological concentrations (10^{-8}-10^{-7} M) did not affect AA release from intracellular stores (Fig. 3). In the case of inflammatory concentrations of AA (10^{-6}-10^{-5} M), the rate of AA release from macrophages was significantly increased. The amount of released radioactivity and the rate of this process became comparable with those observed under stimulation of the cells with the calcium ionophore A23187 (Fig. 3) which is usually used for stimulation of AA release from phospholipids of cell membranes. Thus, the described decrease in the relative fraction of AA incorporated into the cells in the range of inflammatory concentrations can be assumed to be activation of AA release from the phospholipids of cell membranes.

Modeling of AA uptake by the cells. To describe the experimental results and to test our assumption about AA release from intracellular stores of macrophages, we developed a model of AA incorporation into the cells. This process can be divided into the following stages [19, 27]: 1) transport of AA to the cell surface; 2) penetration of AA through the plasma membrane; 3) incorporation into phospholipids.

We omitted the transport stage in our model because in the experimental system used AA was added directly to the cells.

The process of penetration of the cell plasma membrane by AA and other FAs is still not clear. In both mechanisms proposed in the literature, AA is bound to the membrane and then is distributed between the extracellular medium and cells with a characteristic time on the order of seconds [19, 22]. The rates of FA transport across the membrane are significantly higher than observed rates of FA incorporation into cells [19, 27]. In our experiments, the observation was continued for several hours. Thus, we count this penetration through the membrane stage as an equilibrium stage.

We assume that the process of AA uptake by the macrophages (Fig. 1b) described as "rapid" corresponds to redistribution of AA between the culture medium, membranes, and intracellular space and the "slow" one corresponds to incorporation of AA into phospholipids.

Incorporation of AA into phospholipids is a multistep process comprising several stages of successive acylation and deacylation [4]. In most cases, this process limits the overall rate of AA uptake by cells. The observed rate can be used as a characteristic of this process.

It is known that within stimulated cells AA can be oxidized into eicosanoids by cyclooxygenases, lipoxygenases, and other enzymes [28], but even under maximal stimulation, which can be achieved by the action of zymosan, the total amount of these metabolites does not exceed 5-6% of the total content of AA in the cell [29-33]. Therefore, we did not take into account the oxidative metabolism in modeling of AA incorporation into macrophages.

Equilibrium model of AA incorporation into cells. Accepting the assumptions made, the equilibrium process of AA incorporation into macrophages can be described by the scheme:

$$AA + Mem \overset{K_1}{\leftrightarrow} AA_{Mem} \overset{K_2}{\leftrightarrow} AA_{PL}, \quad (1)$$

where AA is arachidonic acid in the extracellular space; Mem is the regions of membrane interacting with AA; AA_{Mem} is AA bound to the membrane and free within the cell; AA_{PL} is AA incorporated into phospholipids; K_1 and K_2 are equilibrium constants.

In our work, we observed the quantity of radioactive label in the medium. All labeled compounds are marked with the symbol "*". In this case, the concentrations of components normalized to the total system volume will be determined by the following equations:

$$K_1 = \frac{[AA][Mem]}{[AA_{Mem}]}, \quad K_1 = \frac{[AA^*][Mem]}{[AA^*_{Mem}]}, \quad (2)$$

$$K_2 = \frac{[AA_{Mem}]}{[AA_{PL}]}, \qquad K_2 = \frac{[AA^*_{Mem}]}{[AA^*_{PL}]}. \quad (3)$$

In the absence of AA in the system before addition of exogenous AA, the mass balance can be written as:

$$[AA]_0 = [AA] + [AA_{Mem}] + [AA_{PL}],$$

$$[AA^*]_0 = [AA^*] + [AA^*_{Mem}] + [AA^*_{PL}], \quad (4)$$

where $[AA]_0$ and $[AA^*]_0$ are initial concentrations of cold and labeled AA.

From the experimental conditions, $[AA^*]_0 << [AA]_0$ and $[AA^*]_0 << [Mem]_0$ ($[Mem]_0$ is the initial concentration of AA binding centers in membrane and inside the cell). Thus, we have:

$$[AA]_0 = [AA] + [AA_{Mem}] + [AA_{PL}]. \quad (5)$$

Substituting $[AA_{Mem}]$ and $[AA_{PL}]$ in (4) for expressions obtained from (2) and (3), we get:

$$[AA]_0 = [AA] + \frac{[AA][Mem]}{K_1} + \frac{[AA][Mem]}{K_1 \cdot K_2}. \quad (6)$$

Thus, [AA] can be described as:

$$[AA] = \frac{[AA]_0}{1 + (1 / K_1 + 1 / K_1 \cdot K_2)[Mem]} \quad (7)$$

or taking $1/K_1 + 1/K_1 \cdot K_2$ as ω, we have:

$$[AA] = \frac{[AA]_0}{1 + \omega\,[Mem]}. \quad (8)$$

Similarly,

$$[AA^*] = \frac{[AA^*]_0}{1 + \omega\,[Mem]}. \quad (9)$$

Thus, for observed parameter $[AA^*]/[AA^*]_0$, which is the relative fraction of the label remaining in the medium, we get:

$$\frac{[AA^*]}{[AA^*]_0} = \frac{1}{1 + \omega\,[Mem]}. \quad (10)$$

By grouping of (2), (3), and (5) we get:

$$[Mem] = \frac{[Mem]_0}{1 + \omega\,[AA]}. \quad (11)$$

Putting (8) into (11), we have:

$$[Mem] = \frac{[Mem]_0}{1 + \dfrac{\omega\,[AA]_0}{1 + \omega\,[Mem]}}. \quad (12)$$

The values of [Mem] in dependence on $[AA]_0$ can be found from (12) as roots of the square equation for [Mem] with linear coefficients containing ω and $[AA]_0$. At $[AA]_0 << [Mem]_0$, we get $[Mem] \approx [Mem]_0$. Thus,

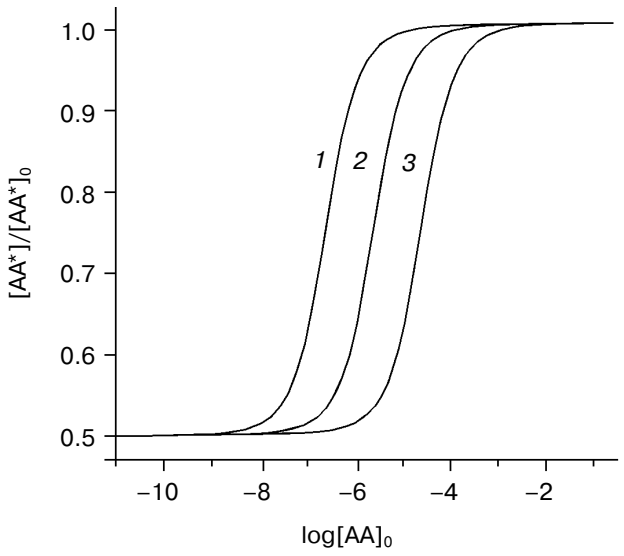

Fig. 4. Theoretical dependence of radioactivity remaining in the medium at equilibrium after introduction into the system the mixture of "cold" AA and [^{3}H]AA (AA*) for scheme (1) with different values of $[Mem]_0$: *1*) 10^{-7}; *2*) 10^{-6}; *3*) 10^{-5}. Conditions: $[AA]_0 >> [AA^*]_0$; $[Mem]_0 >> [AA^*]_0$; $[AA_{Mem}]_0 = 0$; $[AA_{PL}] = 0$.

from experimental data the relative fraction of incorporated AA in the range of low applied concentrations consists of 50% (Fig. 2). From (10) we obtain $\omega = 1/[Mem]_0$. Taking this into account, one can plot the theoretical dependence of the relative fraction of the label in the medium on initial concentration of AA (Fig. 4). As one can see, in the range of $[AA]_0 << [Mem]_0$, this fraction is a constant. Next, if $[AA]_0$ is increased so a larger fraction of the label remains in the medium up to $[AA^*]/[AA^*]_0 = 1$, then the system is saturated by AA.

Let us indicate total content of endogenous AA in experimental system as $[AA_{cell}]$ and concentration of exogenous AA as $[AA_{ex}]$. So,

$$[AA]_0 = [AA_{cell}] + [AA_{ex}] . \qquad (13)$$

Thus, even at $[AA_{ex}] = 0$, the condition $[AA]_0 << [Mem]_0$ may not hold. Nevertheless, at $[AA_{ex}] << [AA_{cell}]$, $[AA]_0 = const$ and the incorporated fraction of AA will be a constant. With the increase of $[AA_{ex}]$ the behavior of the system will fit the one with the previously described condition when $[AA_{cell}] = 0$.

One can see that the theoretical curves of $[AA^*]/[AA^*]_0$ vs. $[AA]_0$ calculated from this model have ranges of AA concentration with two levels of AA uptake (Fig. 3). However, the relative fraction of label remaining in the extracellular space ($[AA^*]/[AA^*]_0$) is always equal to 1 in the range of inflammatory AA concentrations. That result does not fit the experimental data (Figs. 1a and 2), where this parameter is 0.7.

The steady state model of AA uptake into cells. As shown, AA at inflammatory concentrations increases the rate of AA release from the intracellular stores (Fig. 3). We propose that exogenous AA does not saturate AA uptake. In other words [AA] << [Mem] and rate constant of AA release from intracellular stores is changed depending on exogenous AA concentration. Accepting this, scheme (1) can be rewritten as:

$$AA + Mem \underset{}{\overset{K_1}{\leftrightarrow}} AA_{PL} \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} AA_{PL} , \; k_{-2} = f([AA]) , \qquad (14)$$

where k_2, k_{-2} are the observed rate constants of direct and reverse processes of AA incorporation into phospholipids.

Thus, $[Mem] = [Mem]_0 = const$ and [Mem] can be moved into the equilibrium constant:

$$K_1' = \frac{[AA]}{[AA_{Mem}]} , \qquad K_1' = \frac{[AA^*]}{[AA^*_{Mem}]} , \qquad (15)$$

and expression (3) can be substituted for:

$$\frac{k_{-2}}{k_2} = \frac{[AA_{Mem}]}{[AA_{PL}]} , \quad \frac{k_{-2}}{k_2} = \frac{[AA^*_{Mem}]}{[AA^*_{PL}]} . \qquad (16)$$

Then,

$$[AA^*]_0 = [AA^*] + \frac{[AA^*]}{K_1'} + \frac{[AA^*]}{K_1'} \frac{k_2}{k_{-2}} \qquad (17)$$

or

$$\frac{[AA^*]}{[AA^*]_0} = \frac{1}{1 + 1/K_1' (1 + k_2/k_{-2})} . \qquad (18)$$

At high AA concentrations, $k_{-2} >> k_2$, so:

$$\frac{[AA^*]}{[AA^*]_0} = \frac{1}{1 + 1 / K_1'} \qquad (19)$$

and therefore, K_1' value can be calculated from experimental data. Having $[AA^*]/[AA^*]_0 = 0.7$, we get $K_1' = 2.3$. Substituting this value into (19) and accepting $[AA^*]/[AA^*]_0 = 0.5$ under low AA concentrations (Fig. 2), we get $k_2/k_{-2} = 1.33$.

From the expression k_{-2} as a linear function for $[AA]_0$ ($k_{-2} = a[AA]_0 + b$), we can write k_2/k_{-2} as:

$$\frac{k_2}{k_{-2}} = \frac{\beta}{\alpha\,[AA]_0 + 1}, \qquad (20)$$

where $\beta = k_2/k_{-2}$ under low AA concentrations ($\alpha \cdot [AA]_0 << 1$). Or $\beta = 1.33$, and α is a parameter of the system responsible for the beginning of change in the fraction of AA incorporated at the equilibrium state. There are two ranges of concentrations of exogenous AA with different levels of AA uptake on the theoretical curves for this system (Fig. 5a). This theoretical result is adequate for incorporation of AA at normal and inflammatory concentrations into the cells. However, changes in the uptake level proceed during changes in AA concentration with two orders. With this criterion, the theoretical data for this model deviates from experimental data where this change is observed under changing AA concentration by one order of magnitude, from 10^{-7} to 10^{-6} M.

In biological systems, physiologically active compounds activating different processes act as triggers. So, a slight change in concentration can induce significant changes in the rates of processes. In other words, the order of constant rate dependency on concentration of these triggers can be significantly higher than 1. This is true for activation of different receptors and ion channels.

If one assumes that order of the dependence of k_{-2} on AA is 2, then:

$$\frac{k_2}{k_{-2}} = \frac{\beta}{\alpha\,[AA]_0^2 + 1}. \qquad (21)$$

In this case a change between two levels of AA uptake occurs during changes of AA concentrations with one order and theoretical curve at $\alpha = 10^{12}$ fits the experimental data (Fig. 5b). Thus, the model including activation of endogenous AA by exogenous AA is consistent with the experimental results.

Possible reasons for the difference in AA metabolism in the presence of normal and inflammatory concentrations of exogenous AA. As we have shown, exogenous AA at inflammatory concentrations stimulates AA release from intracellular stores. Intracellular metabolism of AA from different sources can be different [6-8, 13, 34]. Therefore,

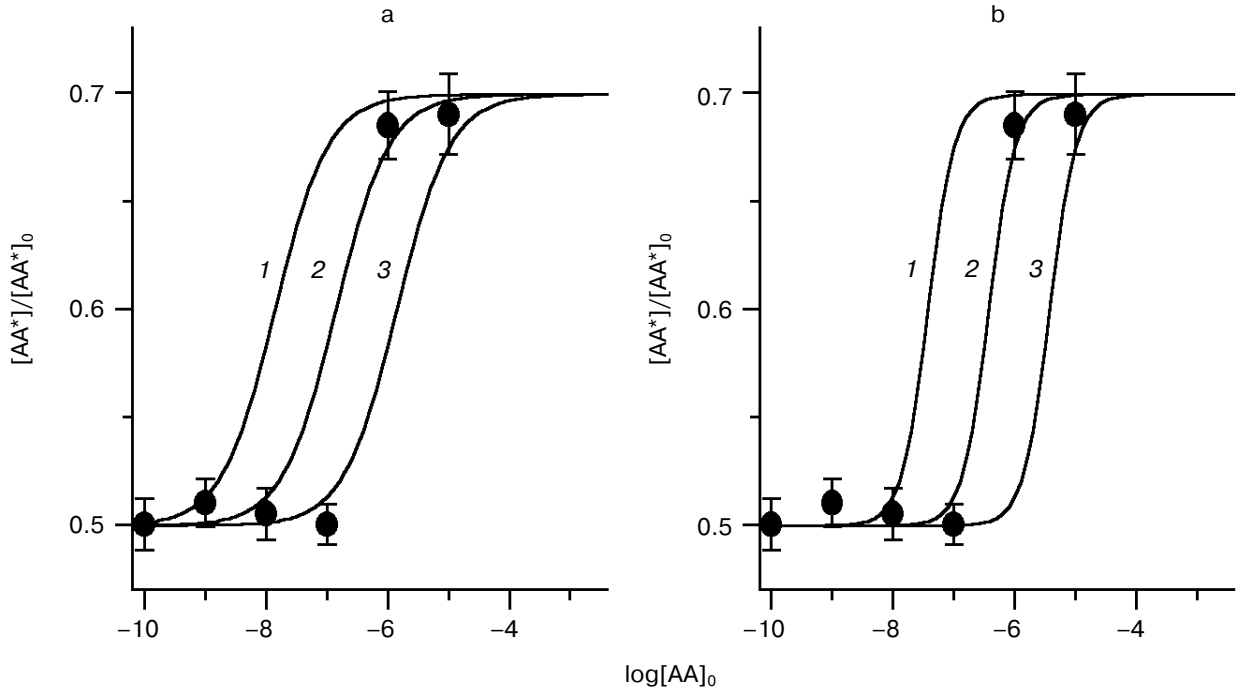

Fig. 5. Comparison of theoretical data and experimental results for the fraction of AA remaining in solution in the steady state after introduction into the system the mixture of "cold" AA and [^{3}H]AA (AA*) for scheme (1) with different values of parameter α: *1*) 10^7; *2*) 10^6; *3*) 10^5 (a); *1*) 10^{14}; *2*) 10^{12}; *3*) 10^{10} (b). The theoretical curves were calculated according to scheme (14):

$\frac{k_2}{k_{-2}} = \frac{\beta}{\alpha\,[AA]_0 + 1}$ (a), $\frac{k_2}{k_{-2}} = \frac{\beta}{\alpha\,[AA]_0^2 + 1}$ (b). Conditions: $[AA]_0 >> [AA^*]_0$; [Mem] >> [AA]; $\beta = 1.33$. Black circles, experimental results. Conditions were as in Fig. 1.

exogenous AA can participate in eicosanoid biosynthesis not only as a substrate, but also as a regulator of this process.

As mentioned above, AA release generally occurs due to phospholipid hydrolysis catalyzed by PLA_2. These enzymes can be activated either by phosphorylation or/and by increase of intracellular Ca^{2+} concentrations [35]. In different cell types, AA is known to affect membrane permeability for different ions, to induce Ca^{2+} release from intracellular stores [36], to influence the activity of different protein kinases [37], and to regulate gene expression [37]. We suppose that exogenous AA in inflammatory concentrations acts as a trigger activating one or several pathways of PLA_2 activation. To clarify the principles of eicosanoid biosynthesis correction under pathophysiological conditions, further investigation of the mechanisms of PLA_2 activation should be useful.

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