The hypolipidemic drug metabolites nafenopin-CoA and ciprofibroyl-CoA are competitive $P2Y_1$ receptor antagonists

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Abstract Coenzyme A (CoA-SH), endogenous and drug-derived CoA-derivatives were tested as putative antagonists of P2Y receptors expressed in *Xenopus laevis* oocytes, a method used to determine calcium-activated chloride current, an indicator of the activation of these receptors. CoA-SH antagonized reversibly and in a concentration-dependent manner the ATP-gated currents evoked by the human $P2Y_1$ but not the $P2Y_2$ receptor. Palmitoyl-CoA was four-fold more potent than CoA-SH as an antagonist while palmitoyl-carnitine was inactive, highlighting the role of the CoA-SH moiety in the antagonism. The CoA derivatives of nafenopin and ciprofibrate, two clinically relevant hypolipidemic drugs, increased 13 and three-fold the potency of CoA-SH, respectively. The K_Bs of nafenopin-CoA and ciprofibroyl-CoA were 58 and 148 nM, respectively; the slopes of the Schild plots were unitary. Neither 100 μM nafenopin nor ciprofibrate alone altered the $P2Y_1$ receptor activity. Neither CoA-SH nor ciprofibroyl-CoA antagonized the rat $P2X_2$ or the $P2X_4$ nucleotide receptors nor interacted with the $5\text{-}HT_{2A/C}$ receptors. The bulky drug CoA-SH derivatives identify a hydrophobic pocket, which may serve as a potential target for novel selective $P2Y_1$ antagonists.

Key words: P2Y1 receptor antagonists; Acyl-CoA derivatives; Palmitoyl-CoA; Fibrates; Hypolipidemic drugs; Nafenopin-CoA; Ciprofibroyl-CoA; Competitive antagonism

1. Introduction

Fibrates such as nafenopin and ciprofibrate have been extensively used as hypolipidemic drugs. The administration of these compounds to experimental animals causes a notable increase in liver peroxisomes. Only recently fibrates, together with a variety of endogenous lipophilic compounds, have been recognized as ligands of the peroxisomal proliferator-activated receptors (PPARs), which are a group of three nuclear receptor isoforms encoded by different genes [1]. Fibrates are metabolized either as acyl-CoA derivatives or as glucuronide conjugates [2,3]. Fatty acids and related lipids are incorporated to the cell metabolism as acyl-CoAs, reaching intracellular concentrations of 5–160 μM; specific acyl-CoA binding proteins and fatty acid binding proteins maintain the levels of free acyl-CoAs in the range of 0.1–200 nM [4]. Upon administration of these compounds to rodents, the intracellular levels of these drug-CoAs may reach high micromolar concentrations [2,4,5], similar to or even higher than those of the endogenous fatty acyl-CoAs [6].

The $P2Y_1$ receptor was the first nucleotide receptor cloned and identified as a member of the family of receptors coupled to G proteins [7]. It was initially cloned from chick brain [8] and immunocytochemical and physiological evidence soon demonstrated its presence in many regions of the human CNS, including the hippocampus, cerebellar cortex, caudate nucleus [9], as well as in the vascular tree [10] and human platelets [11]. In view of the structural similarity between ADP/ATP, the endogenous ligands of the $P2Y_1$ receptor, and coenzyme A (CoA-SH), we investigated whether CoA-SH and/or its acyl derivatives, either endogenously derived from fatty acids or derived from the metabolism of drugs such as the fibrates, antagonize the P2Y receptors. Nafenopin-CoA and ciprofibroyl-CoA were particularly attractive since they have a bulky and hydrophobic acyl substitution, which could help test if bulkiness increases the antagonist potency of endogenous CoAs. For this purpose, we expressed the human $P2Y_1$ receptor in *Xenopus laevis* oocytes and performed electrophysiological protocols in the absence and in the presence of endogenous and exogenous CoA derivatives.

2. Materials and methods

2.1. Materials

ATP, ADP, acyl-CoAs, acyl-carnitines, CoA-SH, 5-HT, MRS 2179, sodium salts, and penicillin–streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The synthesis of Naf-CoA, Cip-CoA and palmitoyl-CoA was performed according to the procedure of chemical synthesis described by Bronfman et al. [12]. The drug CoA derivatives were purified to a single high-performance liquid chromatography (HPLC) peak following several chromatographic conditions; enzymatic hydrolysis and mass spectroscopy certified pure products. Analytical reagents used to prepare the buffers were purchased from either Sigma-Aldrich (USA) or Merck (Darmstadt, Germany).

2.2. Oocyte preparation and expression of receptors

X. laevis oocytes were manually defolliculated and incubated with collagenase; separate batches of oocytes were injected intranuclearly with 0.5 ng cDNA coding for the human $P2Y_1$ or the $P2Y_2$ receptor proteins. Electrophysiological studies were performed as detailed in Acuña-Castillo et al. [13]; ATP-gated currents were recorded following a 10 s nucleotide application. Parallel protocols examined the specificity antagonist of the drug CoA derivatives in rat $P2X_2$,

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$P2X_4$, 5-HT_{2A} or 5-HT_{2C} receptor clones. The P2Y and 5-HT_2 receptor ligands activate phospholipase C, indirectly promoting the release of calcium, which activates a chloride current used as an indicator of receptor activity.

2.3. Antagonism of acyl-CoA and related compounds

To evaluate the relative potency of CoA-SH, Naf-CoA, and other acyl-CoA derivatives as putative receptor antagonists, ATP dissolved in Barth's medium was applied in the absence and later in the presence of 1 nM–10 μM acyl-CoA derivatives, also dissolved in Barth's buffer. The compounds were pre-applied for 15 s, followed by a 10 s co-application with ATP (0.2 μM for the $P2Y_1$ and 3 μM for the $P2Y_2$ receptors). The mean inhibitory concentration (IC_{50}) was interpolated from each curve; results are expressed as the average ± S.E.M.

We assessed the magnitude of the inhibition of the ATP-evoked currents when 1 μM CoA-SH or 1 μM Cip-CoA were pre-applied for 0, 15, 30, and 60 s, followed by a 10 s co-application with ATP. To evaluate the nature of the antagonism, ATP concentration–response curve measurements were performed in the absence and later in the presence of 0.1, 0.3, 1, or 5 μM Naf-CoA or Cip-CoA, pre-applied for 15 s, followed by its co-application with ATP. The same oocyte was used to compare an ATP concentration–response curve in the absence and later in the presence of CoA derivatives. Separate protocols established the selectivity of the receptor blockade in the presence of 10 μM CoA-SH or 10 μM Cip-CoA in $P2Y_2$, $P2X_2$, $P2X_4$, 5-HT_{2A} or 5-HT_{2C} receptors, challenged in each case with the median effective concentration of each ligand. Non-parametric statistical analysis was performed.

Curves were normalized against 10 μM ATP, using the GraphPad 'Prism' program (GraphPad Software, San Diego, CA, USA). From the corresponding Schild plots, pA_2 values and their $-\log(K_B)$ were calculated by regression analysis; the slope of the plots was also estimated. The Kruskal–Wallis test [14] was used as a non-parametric test for statistical analysis; the Student *t*-test for multiple comparisons to a common control (Dunnett's tables) was also used when appropriate.

2.4. Determination of the octanol/buffer partition coefficients

To assess the influence of hydrophobicity on the antagonist potency of the compounds tested, the partition coefficients based on the relative solubility of nafenopin, ciprofibrate, palmitic acid, and acetate in octanol and Barth's buffer were determined. Since the CoA moiety makes the drug more hydrophilic, and because we were particularly interested in the contribution of the bulkiness/hydrophobicity of the drug to the CoA antagonism, we compared the partition coefficients of the drugs in their non-metabolized form. For this purpose, 30 μg of ciprofibrate or nafenopin was dissolved in methanol and mixed in equal parts of octanol and buffer to determine their concentrations in the organic and the aqueous phase. After thorough mixing, an aliquot of each phase was injected into a C_{18} chromatographic column and eluted isocratically with methanol/H_2O in 20 mM KH_2PO_4 buffer (pH = 5.5). This procedure allows the analytical separation and detection of the fibrates in either phase. Control protocols established the linearity of the procedure and optimized the conditions for chromatographic drug quantifications. Likewise, the partition coefficient of acetate and palmitate was determined using a similar procedure except that ^{14}C-labeled standards were used; radioactivity was measured in each phase by dissolving an aliquot of each phase in a standard scintillation buffer. Based on these data, the partition coefficients were calculated.

Table 1
Antagonism of acyl-CoAs and related compounds over the $P2Y_1$ receptor

Drug	IC_{50}, μM (x ± S.E.M.)	n
CoA-SH	0.93 ± 0.17	4
Nafenopin-CoA	0.07 ± 0.01**	4
Nafenopin	> 100	4
Ciprofibroyl-CoA	0.34 ± 0.05*	5
Ciprofibrate	> 100	4
Palmitoyl-CoA	0.23 ± 0.07**	4
Palmitoyl-carnitine	> 100	4
Acetyl-CoA	1.38 ± 0.57	3
Acetyl-carnitine	> 100	4
MRS 2179	0.020 ± 0.018**	4

Oocytes were challenged with 0.2 μM ATP. The compounds were pre-applied for 15 s and then co-applied together with ATP.
*$P < 0.05$; **$P < 0.01$, as compared to the IC_{50} of CoA-SH (Kruskal–Wallis and Dunnett's *t*-test).

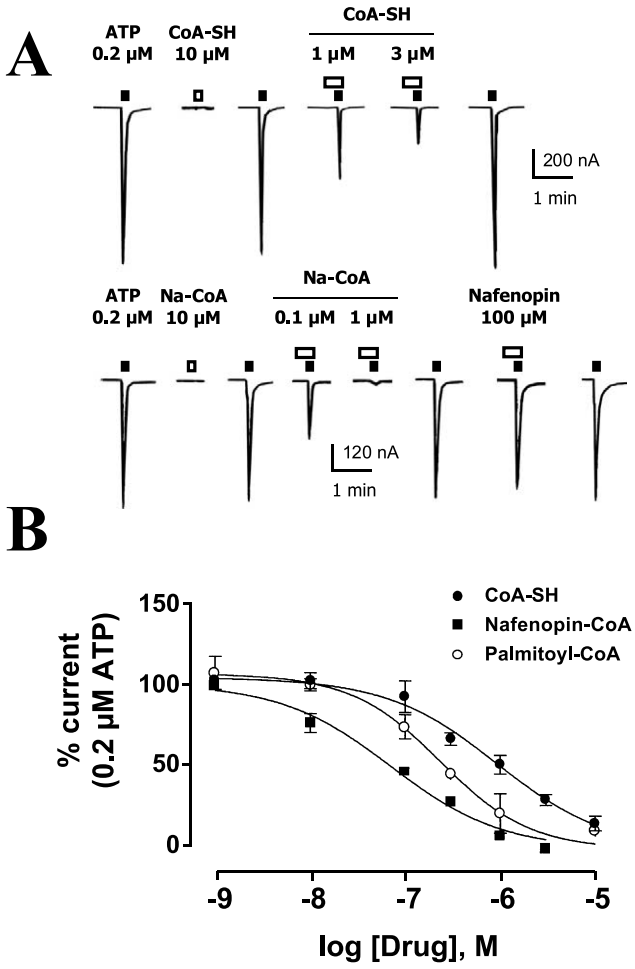

Fig. 1. Antagonism by CoA-related compounds. A: Representative tracings. Neither CoA-SH nor nafenopin-CoA (Na-CoA) evoked any response when applied to an oocyte expressing the $P2Y_1$ receptor, but they inhibited the ATP-evoked currents when pre-applied and then co-applied with the agonist. This effect was not mimicked by nafenopin. These recordings represent calcium-activated chloride currents used as an indicator of nucleotide receptor activity. B: A collection of data showing antagonism induced by CoA-SH (filled circles), nafenopin-CoA (filled squares), and palmitoyl-CoA (open circles) in at least four different oocytes expressing the $P2Y_1$ receptor.

3. Results

3.1. CoA-SH antagonized the currents evoked by ATP in $P2Y_1$ receptors

CoA-SH blocked in a reversible and concentration-dependent manner the current gated by 0.2 μM ATP, a value close to the ligand median effective concentration. The IC_{50} for CoA-SH was 0.93 ± 0.17 μM ($n = 4$, Fig. 1 and Table 1); the sole application of up to 10 μM CoA-SH, in the absence of ATP, did not elicit detectable currents (Fig. 1). MRS 2179, a 3′,5′-bisphosphate purine analog with recognized selectivity

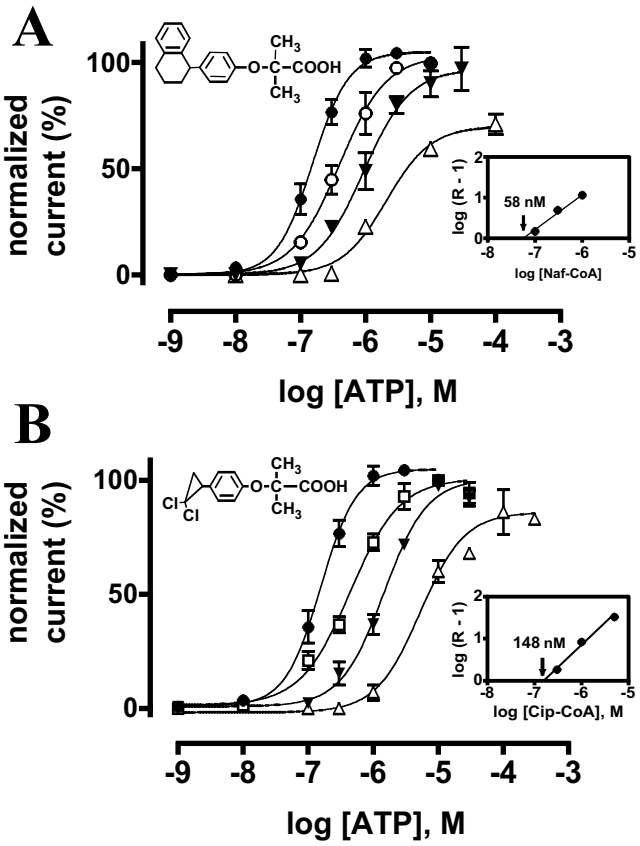

Fig. 2. Nafenopin-CoA and ciprofibroyl-CoA are competitive antagonists of the $P2Y_1$ receptor. A: Concentration–response curves for ATP alone (filled circles) or plus 0.1, 0.3 and 1 μM of nafenopin-CoA (Naf-CoA); the inset shows the Schild plot for these experiments. Naf-CoA was pre-applied for 15 s and then co-applied with ATP, $n=4$. B: Concentration–response curves of ATP in the absence (filled circles) and in the presence of 0.3 (open squares), 1 (filled triangles) and 5 (open triangles) μM of ciprofibroyl-CoA (Cip-CoA); the inset shows the Schild plot for these curves. Cip-CoA was pre-applied and then co-applied with ATP, $n=4$.

for the $P2Y_1$ receptor, had an IC_{50} almost 50-fold lower (Table 1). Increasing the duration of the CoA-SH pre-application from 15 to 30 or 60 s did not further increase the magnitude of the inhibition (data not shown), suggesting that within 15 s the interaction had reached equilibrium.

3.2. Potency and nature of the nafenopin-CoA and ciprofibroyl-CoA-induced $P2Y_1$ receptor antagonism

While Naf-CoA was 13-fold more potent than CoA-SH (Fig. 1), Cip-CoA was only three-fold more active than CoA-SH to block the current evoked by 0.2 μM ATP (Table 1). Neither 100 μM nafenopin nor ciprofibrate altered the ATP-evoked currents (see tracings in Fig. 1 and Table 1). Naf-CoA and Cip-CoA displaced rightwards, and in a parallel manner, the ATP concentration–response curves (Fig. 2), compatible with competitive antagonism. Neither drug-CoAs reduced the maximal ATP-evoked currents except for the largest concentrations of ATP following 1 μM Naf-CoA. The estimated K_Bs for Naf-CoA and Cip-CoA were 58 and 148 nM, respectively. Confirming the competitive nature of the antagonism, the slopes of the corresponding Schild plots were 0.89 and 1.01, respectively.

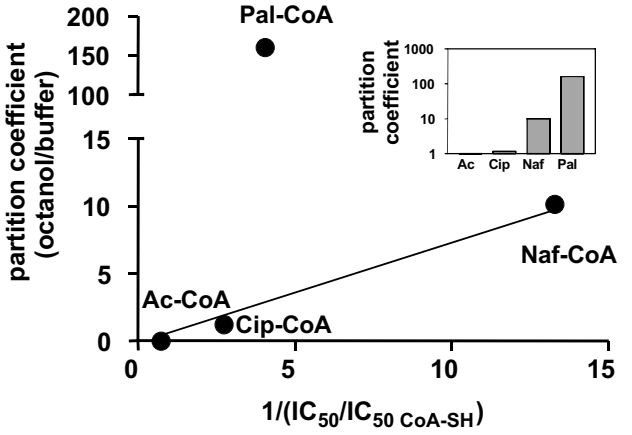

Fig. 3. Octanol/buffer partition coefficients. The relative antagonist potency of acetyl-CoA (Ac-CoA), ciprofibroyl-CoA (Cip-CoA), palmitoyl-CoA (Pal-CoA) and nafenopin-CoA (Naf-CoA) was plotted against the octanol/buffer partition of their hydrophobic substituents shown in the inset (Ac, acetate; Cip, ciprofibrate; Naf, nafenopin; Pal, palmitate).

3.3. Endogenous acyl-CoAs

Acetyl-CoA and palmitoyl-CoA also antagonized the ATP-evoked currents in a reversible fashion (Fig. 1B and Table 1). Acetylating CoA-SH did not modify its relative potency in a significant manner, but palmitoyl-CoA was four times more potent than CoA-SH. 100 μM acetyl or palmitoyl-carnitine were inactive, confirming the importance of the CoA-moiety for the antagonist interaction (Table 1).

3.4. Relationship between partition coefficient and CoA-derivative potency

The octanol/buffer partition coefficient of nafenopin was about 10-fold larger than that of ciprofibrate, and 10 times lower than that of palmitate (Fig. 3, inset). A linear relationship was found between the partition coefficients of acetate, ciprofibrate, and nafenopin and the relative potency of their respective CoA-SH derivatives ($r=0.99$, $P<0.05$, Fig. 3). Palmitate did not follow this linear relationship, indicating that additional factors besides hydrophobicity are important for the interaction with the receptor.

3.5. Subtype selectivity and receptor specificity of drug-CoAs

All the acyl-CoAs examined, including CoA-SH, showed

Table 2
Specificity of antagonism induced by CoA-SH and ciprofibroyl-CoA

Receptor	Control	Percentage of response	
		+10 μM CoA-SH	+10 μM Cip-CoA
$P2Y_1$	100 ± 2.2 (27)	15.8 ± 4.0* (4)	3.1 ± 1.9* (4)
$P2Y_2$	100 ± 2.3 (19)	104.8 ± 7.6 (4)	107.5 ± 13.3 (4)
$P2X_2$	100 ± 3.7 (9)	104.3 ± 12.2 (4)	99.4 ± 7.0 (4)
$P2X_4$	100 ± 1.0 (6)	106.2 ± 3.6 (4)	105.4 ± 1.5 (4)
$5\text{-}HT_{2A}$	100 ± 1.1 (8)	96.2 ± 5.0 (4)	91.9 ± 18.0 (3)
$5\text{-}HT_{2C}$	100 ± 8.5 (4)	101.2 ± 7.9 (3)	101.5 ± 7.5 (4)

Values are mean ± S.E.M.; the number of experiments is indicated in brackets. CoA-SH and ciprofibroyl-CoA (Cip-CoA) were pre-applied for 15 s and then co-applied with the agonist in all cases. Agonists used: 0.2 μM ATP for $P2Y_1$ receptors; 3 μM ATP for $P2Y_2$ receptors; 30 μM ATP for $P2X_2$ receptors; 10 μM ATP for $P2X_4$ receptors; 0.1 μM 5-HT for $5\text{-}HT_{2A}$ receptors and 0.01 μM 5-HT for $5\text{-}HT_{2C}$ receptors. *$P<0.001$, as compared with the currents obtained with ATP alone (Kruskal–Wallis test).

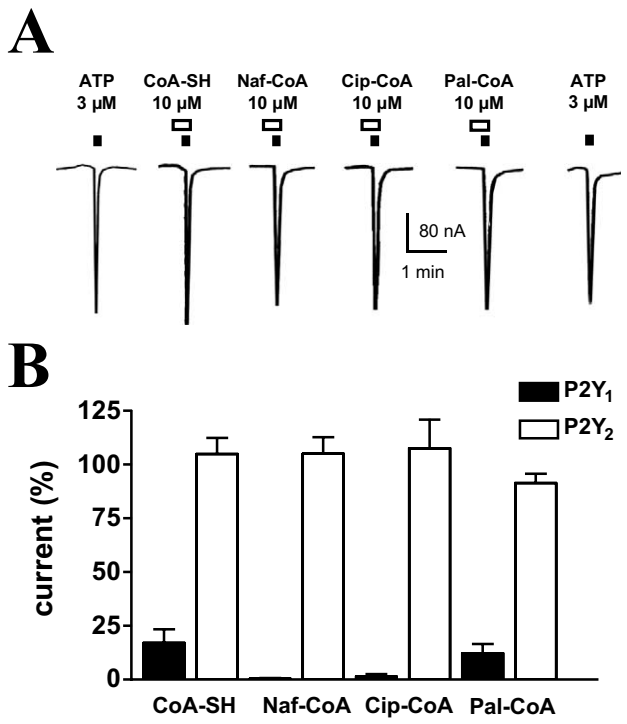

Fig. 4. Selective $P2Y_1$ receptor antagonism induced by drug-CoAs and palmitoyl-CoA. A: Representative tracings of an oocyte expressing the $P2Y_2$ receptor and the effects of CoA-SH, nafenopin-CoA (Naf-CoA), ciprofibroyl-CoA (Cip-CoA) and palmitoyl-CoA (Pal-CoA). B: Comparison of the effects induced by these compounds in $P2Y_1$ (filled bars) and $P2Y_2$ receptors (open bars). 10 μM of CoA derivatives was pre-applied for 15 s and then co-applied with 0.2 μM ATP for $P2Y_1$ receptors and 3 μM ATP for $P2Y_2$ receptors, with the exception of Naf-CoA in $P2Y_1$ receptors; in this case 3 μM Naf-CoA induced a complete inhibition of the ATP-evoked response. Bars represent mean ± S.E.M. of at least four separate oocytes.

receptor specificity for the $P2Y_1$ receptor subtype. None of them reduced the currents elicited by ATP acting on $P2Y_2$ receptors (Fig. 4), nor on the $P2X_2$ and $P2X_4$ receptors (Table 2). Furthermore, neither 10 μM CoA-SH nor Cip-CoA antagonized 5-HT responses in 5-HT_{2A} or 5-HT_{2C} receptors, which, like the $P2Y_1$ receptor, are coupled to the Gq_{11} protein (Table 2).

4. Discussion

The present study clearly demonstrates that CoA-SH, fatty acyl-CoA derivatives and fibrate-CoAs are reversible, selective, and competitive antagonists of the human $P2Y_1$ receptor. To understand the mechanism of the antagonism elicited by CoA-SH and its derivatives, it is necessary to observe the structural similarity between the 3′,5′-nucleotide moiety of CoA-SH and ADP/ATP. In our interpretation, the structures hint at a possible interaction of the CoA-SH moiety with the $P2Y_1$ receptor ligand-binding site. The increased potency of the fatty acyl-CoAs, as observed with palmitoyl-CoA, may be due to an interaction at a hydrophobic pocket close to the ligand-binding domain. We now identify that a bulky hydrophobic extension in the CoA-SH moiety, as present in Naf-CoA, further increases the antagonist potency of CoA-SH. A possible interpretation for this finding is that Naf-CoA and the other lipophilic CoA derivatives tested interact with two distinct sites on the receptor. One site would correspond to the purine binding site, which seems to involve several amino acids of the transmembrane domains 3, 6, 7 [15] and charged amino acids that are part of the extracellular loops 2 and 3 [16]. The second site would be a hydrophobic pocket in the vicinity of the purine site, which would involve hydrophobic amino acid residues to accommodate the lipophilic acyl-substituent. This pocket would help stabilize the interaction of the antagonist to the receptor, but its occupation would not elicit a response per se. Consonant with this interpretation, neither nafenopin, ciprofibrate, acetyl-carnitine, nor palmitoyl-carnitine have a $P2Y_1$ receptor antagonist profile.

Even though palmitoyl-CoA and Cip-CoA have similar IC_{50}s, palmitate is 100-fold more hydrophobic than ciprofibrate. Despite the greater hydrophobicity of palmitate, the flexibility of its aliphatic chain might explain the lesser potency than expected solely from the hydrophobicity analyses. The advantage of determining relative hydrophobicity using the intact compound and not the CoA derivative is the higher resolution, since the more soluble CoA derivatives do not allow us to establish the hydrophobic contribution of each compound. The results with palmitate suggest that while hydrophobicity is important for the added antagonism potency, perhaps bulkiness also plays a critical role. Therefore it is plausible that Naf-CoA, which is bulkier than Cip-CoA and palmitoyl-CoA, accommodates better into this putative hydrophobic pocket. By the same argument, the addition of only two carbons to CoA-SH, as in acetyl-CoA, does not increase the potency of the parent compound, confirming the importance of bulk and hydrophobicity for the increase in the antagonist potency.

MRS 2179 is a selective competitive antagonist for the $P2Y_1$ receptor [17,10]; its potency to antagonize the human $P2Y_1$ receptor agrees with the report of Buvinic et al. [10], but is somewhat different from that described in turkey erythrocytes [17], a finding which could reflect species and methodological variations. Considering the structural similarity of MRS 2179 to the endogenous ligands of the $P2Y_1$ receptor, and the lack of a hydrophobic side chain, it is probable that MRS 2179 binds exclusively to the purine-binding site, as is probably the case for the recently developed MRS 2279, a new and more potent 3′5′-bisphosphate purine analog with $P2Y_1$ antagonist properties [18]. This interpretation reinforces the idea that the hydrophobic substituents of CoA derivatives bind to an additional pocket in the vicinity of the nucleotide binding site and that their major effect is to stabilize the interaction of the CoA moiety to the receptor. By the same argument, the adenosine-5′-carboxylic acid derivatives (AdoCAsp conjugates) are considerably less potent as $P2Y_1$ antagonists than the hypolipidemic drug-acyl-CoAs described in this communication, since they lack phosphate groups affecting their affinity for the purine binding site, and a hydrophobic side chain to stabilize the interaction [19]. Along the same lines, Boyer et al. [20] showed that the potency of saturated fatty acyl-CoAs as $P2Y_1$ receptor antagonists increases almost 100-fold with chain length, reaching a cutoff at C12. These results are consonant with our finding that the length of the fatty acid chain contributes to hydrophobicity but requires bulkiness as well to reach a higher potency. Differences in the inhibitory potency of CoA-SH or the endogenous acyl-CoAs examined were found compared to those of Boyer et al.

[20]; these discrepancies might be related in part to differential levels of receptor expression, the intracellular messenger determined and/or in part to differences between the human and the turkey receptor. Boyer et al. [20] reported that CoA-SH behaved as a partial agonist at mM concentrations; in our study 100 μM was inactive and larger concentrations were not tested.

The specificity of drug action is accounted for by the finding that none of the CoA derivatives interact with the ATP binding site in the P2X receptors, which are ionic channels, and are therefore structurally unrelated to the P2Y receptor family. Experiments with 5-HT_2 receptors indicate that these compounds probably interact with the $P2Y_1$ protein, and not with the signaling pathway, since both the $P2Y_1$ and the 5-HT_2 receptors are coupled to Gq_{11} and activate a Ca^{2+}-dependent chloride channel. Since one of the endogenous ligands of the $P2Y_2$ receptor is a pyrimidine [7], it is possible that CoA-SH or acyl-CoA derivatives do not have sufficient affinity for the pyrimidine/purine binding site, and are therefore inactive, explaining the lack of response in these receptors.

The issue of the physiological ligand of the $P2Y_1$ receptor is a matter of debate. Some authors argue that ADP rather than ATP is the endogenous ligand [21–23], while other investigators have shown that ATP antagonizes the $P2Y_1$ receptor, particularly in platelets [11,24]. In our oocyte studies, ATP behaves as an agonist with a median effective concentration of 156 ± 18 nM ($n = 10$). To avoid ATP degradation, we used freshly prepared ATP solutions; HPLC analysis showed no detectable ADP contamination (data not shown). The 10-s ATP application reduces the possibility of its degradation to ADP.

Although it is highly unlikely to find high levels of extracellular CoA-fibrates or fatty acid CoAs due to the abundance of plasma lipases, we are aware that in pathophysiological conditions such as hypoxia, heart ischemia or the presence of necrotic tissue, there is a massive release of intracellular acyl-carnitines from the kidney or the liver [25,26], an indication that intracellularly stored lipids might be released to the plasma. At present there are no determinations of acyl-CoAs, including the fibrate-CoAs in extracellular fluids (Bar-Tana, personal communication). However, during ischemia, considerable changes in heart and brain acyl-CoAs occur [27,28], making it interesting to study the potential role of these endogenous compounds as $P2Y_1$ antagonists during these events. Likewise, fatty acyl-CoAs enhanced glutamate release from rat hippocampal slices [29], a datum that suggests that brain acyl-CoAs may play a role in neuronal plasticity, such as in long-term potentiation. Furthermore, chronic treatment with clofibrate, another clinically used fibrate [30,31], caused platelet aggregation deficiencies, a process in which the $P2Y_1$ receptor seems to play a relevant role [32]. In light of this finding, and keeping in mind that human platelets express the $P2Y_1$ receptor [11] and metabolize drugs to CoA derivatives [2], the present results might have clinical relevance. Additionally, fibrates may also affect coagulation by inhibiting hepatic fibrinogen synthesis, an effect mediated by PPARα [33].

These results may lead to the search of putative long-chain fatty acyl-CoAs as possible endogenous modulators of the $P2Y_1$ receptor, compounds which may mimic the action of these fibrate-CoAs. This hypothesis might offer novel avenues of clinically relevant research. Independent of the clinical relevance of these fibrate metabolites, drug acyl-CoAs, such as Naf-CoA, might be a promising lead to develop novel nucleotide receptor tools, which may help to identify for example the ATP binding pocket or the site of action of putative endogenous modulators.

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