Aspirin Hydrolysis in Human and Experimental Animal Plasma and the Effect of Metal Cations on Hydrolase Activities

Fatma Goksin Bahar and Teruko Imai

Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

Received March 5, 2013; accepted May 6, 2013

ABSTRACT

The hydrolyzing properties of plasma esterases for aspirin were investigated in human plasma and plasma from experimental animals. The observed rates of aspirin hydrolysis were in the following order: rabbit > human > monkey > rat > mouse > dog > minipig. In human, monkey, and dog plasma, aspirin was hydrolyzed by their major hydrolases, paraoxonase (PON), butyrylcholinesterase (BChE), and albumin. In rabbit, mouse, and rat plasma, carboxylesterase (CES) was determined to be the enzyme responsible for aspirin hydrolysis, and in mouse and rat plasma, especially the latter, hydrolase activity was increased by the addition of ethopropazine, a specific inhibitor of BChE. Interestingly, divalent cations affected the plasma activity by enhancing or inhibiting the hydrolase activity of plasma BChE. The addition of 2 mM calcium increased the hydrolysis of aspirin in human, monkey, and dog plasma by 2.7-, 1.9-, and 2.3-fold, respectively. Magnesium showed a similar but lesser effect. Increasing concentrations of calcium and magnesium resulted in a two-phase stimulatory effect on aspirin hydrolysis in human plasma. In contrast, the addition of zinc had an inhibitory effect on plasma BChE activity. It is postulated that calcium and magnesium bind to BChE and thereby change the conformation of the enzyme to a more appropriate position for aspirin hydrolysis.

Introduction

Plasma esterases play an important role in the metabolic detoxification and/or activation of ester-linked compounds and xenobiotics, including organic phosphates (Satoh and Hosokawa, 1998; Imai, 2006; Liederer and Borchardt, 2006; Lockridge and Quinn, 2010). Major esterases in plasma are butyrylcholinesterase (BChE; EC 3.1.1.8), paraoxonase (PON; EC 3.1.1.31), and carboxylesterase (CES; EC 3.1.1.1). The most abundant plasma protein is albumin, which occasionally shows hydrolyzing activity (Li et al., 2005; Berry et al., 2009; Bahar et al., 2012). Acetylcholinesterase (AChE; EC 3.1.1.7) is also present in plasma, but only in negligible amounts (Li et al., 2005).

Esterified compounds are hydrolyzed by these major plasma proteins via different catalytic mechanisms. CES and BChE hydrolyze various compounds, with a catalytic triad consisting of Ser, Glu, and His (Nicola et al., 2003; Imai, 2006; Liederer and Borchardt, 2006; Satoh and Hosokawa, 2006), whereas hydrolysis by PON occurs via a catalytic dyad consisting of two His molecules, and requires divalent calcium for its activation (Kuo and La Du, 1995, 1998; Josse et al., 2001; Harel et al., 2004). Unlike CES, BChE, and PON, albumin acts as a plasma carrier protein by specific and nonspecific binding (Chuang and Otagiri, 2006) and shows esterase-like activity by irreversible acylation of one or more of 82 acetylated residues (Lockridge et al., 2008).

We recently reported on the identification of plasma esterases and their substrate-specific hydrolase activities in human plasma and the plasma of a number of experimental animals (Bahar et al., 2012). We have shown that the content of major plasma esterases varies between different species, resulting in species differences in hydrolase activity. PON and BChE are ubiquitous, and are especially highly expressed in primates and dogs (Bahar et al., 2012). CES is present in a limited number of species, including rabbits, mice, and rats, in which it is an abundant plasma protein, involved in the rapid hydrolysis of most esterified compounds (Bahar et al., 2012). In human plasma, as well as that of monkeys and dogs, several substrates are slowly hydrolyzed by their main esterases, PON and BChE. Interestingly, besides these well known four proteins, several unknown proteins with hydrolyzing activity are also found in minipig and mouse plasma. Despite this, minipig plasma shows an extremely low hydrolyase activity for various substrates, although it has a high activity for β-nitrophenylacetate (PNPA). The differential expression of esterases and their substrate specificities in various animals needs to be studied in more detail, using an appropriate model substrate, to characterize these plasma esterases.

Aspirin is the most widely used drug in the world (Halushka and Halushka, 2002; Vane and Botting, 2003) and it is hydrolyzed to salicylic acid by most esterases, and even by albumin (Morikawa et al., 1979). After oral administration in humans, aspirin undergoes hydrolysis in the intestine, liver, and plasma (Rowland et al., 1972; Williams et al., 1989). Various specific hydrolases play a role in this hydrolysis in different tissues. For example, human CES 1 and 2 are the major contributors to this activity in the liver and intestine, respectively (Inoue et al., 1980; Heymann and Mentlein, 1988; Williams et al., 1989; Tang et al., 2006). In plasma, aspirin is rapidly hydrolyzed (Rowland et al., 1972) by BChE (Hofstee, 1951; Morgan and Truitt, 1965), PON (Santanam and Parthasarathy, 2007; Jaichander et al., 2008), and albumin (Morikawa et al., 1979; Yang et al., 2007). However, their relative contribution to aspirin hydrolysis is still unclear and there are no reports on species differences in the main hydrolases responsible for aspirin hydrolysis in plasma.

ABBREVIATIONS: BChE, butyrylcholinesterase; BNPP, bis-p-nitrophenyl phosphate; CES, carboxylesterase; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; HSA, human serum albumin; PON, paraoxonase.
In this study, the hydrolyzing properties of plasma esterases for aspirin were investigated in several animal species. To clarify the plasma esterases responsible for aspirin hydrolysis, experiments were conducted in the presence of enzyme-specific inhibitors. In addition, the effects of divalent cations, such as calcium, magnesium, and zinc, on aspirin hydrolysis were investigated.

Materials and Methods

Materials

Aspirin (acetylsalicylic acid), salicylic acid (2-hydroxybenzoic acid), bis-p-nitrophenyl phosphate (BNPP), di-sodium dihydrogen ethylenediamine tetraacetate dihydrate (EDTA 2Na dihydrate), calcium chloride dihydrate (CaCl₂ 2H₂O), magnesium chloride hexahydrate (MgCl₂ 6H₂O), and zinc chloride (ZnCl₂) were purchased from Nacalai Tesque (Kyoto, Japan). Purified human serum albumin (HSA) (containing fatty acids) and ethopropazine were from Sigma-Aldrich (St. Louis, MO). Other chemicals and reagents were of analytical grade.

Plasma Samples

Blood was taken from human volunteers (men, 22–26 years old), mice (ddY, male, 10 weeks old), and rats (Wistar, male, 8 weeks old) into heparinized syringes and centrifuged at 1500 × g for 10 min. Studies involving human samples were approved by the Institutional Review Board of Kumamoto University, Faculty of Life Sciences. Written informed consent was signed by healthy human volunteers prior to their participation in the study. Plasma from cynomolgus monkeys (male, 3–4 years old), dogs (Beagle, male, 1 year old), and rabbits (Japanese white strain, male, 10 weeks old) was kindly provided by Sekisui Medical Co., Ltd. (Tokyo, Japan). Minipig plasma (Claw mini-pig, 13 months old) was a kind gift from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). Plasma samples were kept at −80°C until used. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University.

Hydrolysis Experiments

Reaction Conditions. Aspirin solutions were prepared freshly just before the experiments were started by dissolving aspirin in HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (50 mM, pH 7.4). Plasma samples from human and other species were diluted to appropriate concentrations (20%–25%) with HEPES buffer (50 mM, pH 7.4) and aliquots (100 μl) were preincubated at 37°C for 5 min. Reactions were started by the addition of aspirin dissolved in HEPES (100 μl, final concentration 2.5 mM). After 30-min incubation, reactions were terminated by the addition of 400 μl of ice-cold acetonitrile. The reaction mixture was centrifuged at 1600 × g for 10 min, and an aliquot (400 μl) of the supernatant was added to 10% (v/v) phosphoric acid (H₃PO₄, 10 μl) and analyzed by high-performance liquid chromatography (HPLC). The initial hydrolytic activity was measured under reaction conditions in which <20% of substrate was hydrolyzed. The rate of hydrolysis was determined by subtracting the hydrolysis rate in HEPES buffer from that in diluted plasma samples. The hydrolytic activity is given as the concentration of the hydrolysis product.

Effect of Enzyme-Specific Inhibitors. Hydrolysis experiments were repeated in the presence of the following inhibitors: BNPP, ethopropazine, and EDTA at concentrations of 0.5 mM, 0.5 mM, and 1 mM, respectively. BNPP and ethopropazine were dissolved in dimethyl sulfoxide (DMSO) while EDTA was dissolved in HEPES buffer (50 mM, pH 7.4). Diluted plasma samples were incubated with these solutions for 5 min at 37°C before the addition of the substrate and were then processed as above. The final DMSO concentration was less than 1%, which has no effect on hydrolytic activity (Yoshigae et al., 1999; Imai et al., 2006; Bahar et al., 2012). Control reactions in plasma were carried out in parallel by adding solvent only (DMSO or HEPES buffer, 50 mM, pH 7.4). The inhibition percentage (%) was calculated as the relative activity in the presence of the inhibitor compared with control activity.

Effect of Divalent Cations. We investigated the effect of the divalent cations, calcium (Ca), magnesium (Mg), and zinc (Zn), on the hydrolysis of aspirin. CaCl₂, MgCl₂, and ZnCl₂ were dissolved in HEPES buffer (50 mM, pH 7.4) to obtain concentrations of 2–25 nM when mixed with aspirin solutions. The reaction was started by the simultaneous addition of aspirin and divalent cation to preincubated plasma samples. Reaction mixtures were then processed as above.

Evaluation of Kinetic Behavior in the Presence of Divalent Cations. The Michaelis–Menten behavior of the activation in the presence of divalent cations at constant aspirin concentrations was described by deriving an equation based on the model in Scheme 1, where E is the enzyme, S is the substrate (aspirin), M is the metal ion (i.e., divalent cation), ES is the enzyme–substrate complex, EM is the enzyme–metal complex, ESM is the enzyme–substrate–metal complex, and P is the product. K₉ and K₈ are the Michaelis–Menten constants for EM and ESM, respectively, whereas K₆ and K₇ are the dissociation constants for ES and ESM, respectively. k and k’ are the rate constants for the breakdown of ESM to E and P, and ES+M to E and P, respectively.

\[
\begin{align*}
E + S + M & \iff K_6 \iff ES + M \\
EM + S & \iff K_9 \iff ESM \\
EM + S & \iff K_6 \iff ES + M \\
EM + S & \iff K_9 \iff ESM \iff k \iff E + P
\end{align*}
\]

Scheme 1. Model to describe the Michaelis–Menten behavior of the activation in the presence of divalent cations at constant aspirin concentrations.

It is assumed that EM and ESM occur independently and the activity of ES is much lower than that of ESM. Additionally, in the breakdown of ESM, k’, is assumed to be much lower than k. Also, K₉ · K₆ is assumed to be equal to k’ · K₈. Under these assumptions, the initial velocity (v) of activation can be written as

\[
v = k \cdot [ESM],
\]

\[
\text{and the limiting maximal velocity (V}_{\text{max}} \text{) can be taken as } V_{\text{max}} = k \cdot [E][S] \text{, where } [E][S] \text{ represents total enzyme concentration ([E][S] = [E] + [EM] + [ESM] + [ES]). In this way, a model that resembles the Michaelis–Menten equation can be derived, as shown in Eq. 1.}
\]

\[
v = V_{\text{max}}[M][S] / [K_6([M] + [S]) / K_9 + [S] / K_9 + [M]]
\]

This equation describes the rate of the enzymatic reactions, by relating the reaction rate, v (hydrolysis activity) to [M], the concentration of divalent cation.

To represent the enzyme kinetics graphically a model equation (Eq. 2), which resembles the Eadie–Hofstee diagram, is derived from Eq. 1 with reaction velocity (v) plotted as a function of velocity/divalent cation concentration ratio (v/[M]).

\[
\]

the y-intercept, and K₉ and K₈ as the slope.

HPLC Conditions

The concentration of the hydrolysate, salicylic acid, was determined by HPLC. The HPLC system comprised a PU-980 pump, a AS-950 autosampler, a UV-2075 Plus UV detector, a FP-1520 fluorescence detector, a LC Net II/ADC System (all from JASCO Co., Tokyo, Japan), and a 655A-52 column oven (HITACHI Ltd., Tokyo, Japan). For determination, a TSK-GEL ODS-80Ts column (5 μm, 4.6 mm, 150 mm inner diameter; TOSOH Bioscience, Tokyo, Japan) was used. The temperature of the column was maintained at 40°C. Aspirin was detected by UV at 277 nm, whereas salicylic acid was detected by fluorescence at excitation and emission wavelengths of 296 and 405 nm, respectively. During hydrolysis activity experiments, a mobile phase of 0.1% H₃PO₄/acetonitrile (60:40, v/v) was used at a flow rate of 1.0 ml/min. The retention times of aspirin and salicylic acid were 3.2 and 4.4 min, respectively. A mobile phase of 0.1% H₃PO₄/acetonitrile (70:30, v/v) at the same flow rate was used for the inhibition experiments. The retention times of aspirin and salicylic acid were 5.0 and 7.2 min, respectively. The detection limit, which represents the injection amount on the column, was 3 pmol and was measured in a quantitatively linear range.
Statistical Analysis
All results are expressed as the mean ± S.D. of triplicate (n=3) measurements. Data were analyzed using the t test with P < 0.05 as the minimal level of significance.

Results
Hydrolysis of Aspirin in Animal Plasma
Aspirin hydrolysis was analyzed in plasma from human and other animals. In addition, 4% HSA was used to evaluate the effect of albumin on aspirin hydrolysis in human plasma. Hydrolase activities in the plasma of all tested species were verified by the hydrolysis of PNPA, the control substrate. A rapid rate of hydrolysis was obtained in minipig and then rabbit plasma, and the rate then decreased in the following order: mouse > human > dog > monkey > rat (Bahar et al., 2012).

The results of aspirin hydrolysis are shown in Table 1. The hydrolitic activity is represented as the rate in 1 ml intact plasma. The HSA used in our experiments, which contained fatty acid, showed relatively high hydrolase activity, close to that of fatty acid–free HSA (unpublished data). The highest hydrolase activity for aspirin was observed in rabbit plasma, which was approximately 2-fold higher than that in human plasma. The rate of aspirin hydrolysis in human plasma was nearly the same as in monkey plasma (21.6 ± 0.81 and 19.5 ± 2.14 nmol/min per milliliter, respectively). Similar but lower hydrolase activities were observed in mouse and rat plasma. Dog and minipig plasma show the lowest hydrolase activities, with minipig plasma showing nearly the same activity as 4% HSA.

Effect of Enzyme-Specific Inhibitors on Aspirin Hydrolysis
Inhibition experiments were performed to identify the plasma esterases responsible for aspirin hydrolysis. BNPP and ethopropazine were used as specific inhibitors of CES (Block and Arndt, 1978; Yoshigae et al., 1999; Imai et al., 2006) and BChE (Bayliss and Todrick, 1956; Saxena et al., 1997), respectively. EDTA, a chelating agent, was used as an inhibitor of PON, because plasma PON requires calcium ions to maintain its structure and for its esterase activity (Kuo and La Du, 1995; Khersonsky and Tawfik, 2006).

Table 1 shows the effect of the various inhibitors on the hydrolysis of aspirin in the plasma of different animal species. Because human, monkey, dog, and minipig plasma do not contain CES (Li et al., 2005; Bahar et al., 2012), inhibition experiments with BNPP were not performed in these species. In human plasma, the hydrolitic activity of aspirin was decreased by 50% in the presence of EDTA and 30% by ethopropazine, suggesting that PON had a substantial effect with a lesser contribution from BChE. The plasma hydrolase activities of monkey and dog were inhibited by EDTA, suggesting the predominant role of PON on these activities. The hydrolase activity of minipig plasma was inhibited only by ethopropazine, but this inhibition was not statistically significant.

In the species that express CES in their plasma (i.e., rabbit, mouse, and rat), only 10%–15% of hydrolase activity remained after inhibition by BNPP, suggesting that CES is the major enzyme responsible for hydrolysis of aspirin in these species. As we previously reported, in most cases, plasma CES activity is much higher than that of any other plasma esterases (Bahar et al., 2012). Even in the case of aspirin, which is only slowly hydrolyzed in plasma, hydrolysis was primarily due to CES in mouse and rat plasma. Interestingly, hydrolase activity in mouse and rat plasma was significantly increased by the addition of ethopropazine, a specific inhibitor of BChE, whereas hydrolase activity was decreased in rabbit plasma (Table 1). From this result, we postulate that ethopropazine has a positive effect on rodent CES activity. To clarify this hypothesis, we added first ethopropazine and then BNPP to activated CES. As shown in Table 1, the hydrolitic activity, which is increased by the addition of ethopropazine to mouse and rat plasma, was inhibited by the further addition of BNPP.

Calcium Effect on Aspirin Hydrolysis
As mentioned above, aspirin is a well known substrate that is rapidly hydrolyzed by human plasma BChE and albumin. However, inhibition experiments with EDTA also confirmed the contribution of plasma PON to aspirin hydrolysis in human, monkey, dog, and rabbit plasma. To further investigate the role of PON, aspirin hydrolysis experiments were conducted in which calcium was added to the reaction mixture.

The effect of calcium on aspirin hydrolysis was tested at two different concentrations (2 and 25 mM). The low calcium concentration is assumed to be equivalent to the mean free calcium concentration under physiologically normal conditions (1.1–1.4 mM) and including such disease states as hyperthyroidism, cancer with bone metastases, and so forth (all <2.5 mM). As shown in Fig. 1, the simultaneous addition of aspirin and calcium (see Materials and Methods) had a positive effect on hydrolase activities in human plasma and that of other species. Whether aspirin and calcium were added to plasma as separate and/or mixed solutions, similar effects were observed on hydrolase activities (unpublished data). In human, monkey, and dog plasma, the addition of calcium even at this low concentration (2 mM)

### Table 1

Aspirin hydrolysis by HSA and plasma of human and several animals and the effect of esterase inhibitors EDTA, BNPP, and ethopropazine on their hydrolase activities

<table>
<thead>
<tr>
<th>Species</th>
<th>Hydrolase Activity in Controls</th>
<th>Hydrolase Activity in the Presence of Esterase Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM EDTA</td>
<td>0.5 mM BNPP</td>
</tr>
<tr>
<td>HSA (4%)</td>
<td>4.82 ± 0.15</td>
<td>3.96 ± 0.46 (82.1 ± 9.47)</td>
</tr>
<tr>
<td>Human</td>
<td>21.6 ± 0.81</td>
<td>10.7 ± 0.63 (49.5 ± 5.50)***</td>
</tr>
<tr>
<td>Monkey</td>
<td>19.5 ± 2.14</td>
<td>12.3 ± 1.60 (63.2 ± 5.54)*</td>
</tr>
<tr>
<td>Dog</td>
<td>7.51 ± 1.09</td>
<td>5.13 ± 1.46 (68.3 ± 8.67)*</td>
</tr>
<tr>
<td>Minipig</td>
<td>4.79 ± 1.66</td>
<td>4.83 ± 0.20 (101 ± 3.12)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>37.0 ± 2.58</td>
<td>25.4 ± 4.60 (86.8 ± 11.6)*</td>
</tr>
<tr>
<td>Mouse</td>
<td>12.3 ± 4.68</td>
<td>12.0 ± 0.37 (98.6 ± 15.6)</td>
</tr>
<tr>
<td>Rat</td>
<td>15.5 ± 2.81</td>
<td>16.0 ± 2.03 (103 ± 4.68)</td>
</tr>
</tbody>
</table>

* 0.05 < *p < 0.01; **p < 0.001.
increased hydrolytic activity by 2.7-, 1.9-, and 2.3-fold, respectively, whereas high calcium concentrations (25 mM) increased hydrolase activities more than 5-fold. On the other hand, minipig, rabbit, mouse, and rat plasma hydrolase activities were only slightly increased by the addition of 2 mM calcium, whereas they were noticeably increased by the addition of 25 mM calcium except for rabbit plasma.

To confirm the involvement of PON in the increased plasma activity observed in the presence of calcium, inhibition experiments using BNPP and ethopropazine were conducted in the presence of 25 mM calcium. The results are shown in Table 2. The increased activities in human, monkey, and dog plasma in the presence of calcium were effectively inhibited by ethopropazine, resulting in similar levels of hydrolase activities to those seen after inhibition by ethopropazine in the absence of calcium (Tables 1 and 2). These data indicate that BChE is the principle esterase responsible for calcium-activated aspirin hydrolysis in the plasma of these species. In the presence of 25 mM calcium, 48% and 38% of the hydrolase activity remained in mouse and rat plasma, respectively, after the inhibition of CES by BNPP. When BNPP and ethopropazine were added simultaneously, almost complete inhibition was observed in both species. The activating effect of ethopropazine on plasma CES was greater in the presence of 25 mM calcium than in its absence (Tables 1 and 2). Taken together, these results suggest that BChE contributes to the hydrolysis of aspirin, even in mouse and rat, in the presence of calcium.

Effect of Other Divalent Cations on Aspirin Hydrolysis in Human, Monkey, and Dog Plasma

The addition of calcium perceivably increased aspirin hydrolysis in the plasma of all tested animals except minipig and rabbit, mostly by stimulating their BChE activity (Fig. 1; Table 2). To determine the role of other divalent cations on plasma hydrolase activities, further experiments were conducted using human, monkey, and dog plasma in the presence of magnesium and zinc. The results are shown in Fig. 2. The addition of magnesium at the same concentration as calcium (2 mM) increased plasma hydrolase activities in human, monkey, and dog plasma 2.1-, 1.6-, and 1.5-fold, respectively. In contrast, the hydrolysis of aspirin was decreased after the addition of zinc (2 mM). To confirm the esterase responsible for the increased aspirin hydrolysis, inhibition experiments were again performed using ethopropazine in the presence of 2 mM magnesium. In this experiment, the increased hydrolase activities of human, monkey, and dog plasma were inhibited by 80%, 50%, and 63%, respectively (unpublished data). Based on these data in human, monkey, and dog plasma, BChE is the esterase responsible for the increase in aspirin hydrolysis in the presence of calcium and magnesium.

<table>
<thead>
<tr>
<th>Species</th>
<th>Hydrolase Activity in Controls (nmol/min per milliliter plasma)</th>
<th>Hydrolase Activity in the Presence of Esterase Inhibitors (nmol/min per milliliter plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mM BNPP</td>
<td>0.5 mM Ethopropazine</td>
</tr>
<tr>
<td>Human</td>
<td>149 ± 9.42</td>
<td>20.6 ± 8.83 (13.9 ± 6.23)**</td>
</tr>
<tr>
<td>Monkey</td>
<td>75.6 ± 11.7</td>
<td>20.9 ± 7.07 (27.6 ± 6.42)**</td>
</tr>
<tr>
<td>Dog</td>
<td>49.9 ± 5.49</td>
<td>14.1 ± 5.19 (28.0 ± 4.27)***</td>
</tr>
<tr>
<td>Mouse</td>
<td>65.5 ± 0.99</td>
<td>31.2 ± 0.30 (47.7 ± 3.29)**</td>
</tr>
<tr>
<td>Rat</td>
<td>37.2 ± 6.09</td>
<td>12.1 ± 0.92 (37.9 ± 23.7)*</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001.

**TABLE 2**

The effect of BNPP and ethopropazine on aspirin hydrolysis in the presence of 25 mM calcium

Control refers to hydrolysis conditions in the presence of calcium at a constant concentration of aspirin (2.5 mM). Data show statistically significant differences from the control activity observed in the absence of inhibitors. Values represent the mean ± S.D. (n = 3). Values in parentheses represent the remaining hydrolase activities (%) after inhibition by the relevant inhibitor.
Kinetics of the Divalent Cation Effect on Aspirin Hydrolysis in Human Plasma

A further experiment was conducted to examine the effect of divalent cations on aspirin hydrolysis in human plasma at different concentrations of calcium and magnesium. Figure 3, A and B, shows the effects of increasing divalent cation concentrations (1–10 mM) on the hydrolytic activity of human plasma. Increasing concentrations of both calcium and magnesium activated aspirin hydrolysis in human plasma (Fig. 3A). When these data are replotted as hydrolase activity versus hydrolase activity over divalent cation concentration (v versus v/[M]; Fig. 3B), two bisecting straight lines were obtained for the effects of calcium and magnesium on hydrolase activity. The slopes of these plots were related to the binding affinity ($K_M$) of the divalent cations for the esterase enzyme responsible for aspirin hydrolysis (Eq. 2 in Materials and Methods). We supposed that one of the linear relations occurred at calcium concentrations below 4 mM ($K_M = 1.46$ mM). The second linear relation occurred at higher calcium concentrations (4–10 mM) with lower binding affinity ($K_M = 5.65$ mM). Similar results were obtained for magnesium, which showed a high binding affinity ($K_M = 1.00$ mM) and a lower binding affinity ($K_M = 3.02$ mM).

Discussion

As previously reported, various proteins with esterase activity are present in the plasma of the animals tested in this study (Bahar et al., 2012). Both PON and BChE are expressed in the plasma of all of these animals, together with albumin. BChE content is high in human, monkey, and dog plasma, moderate in minipig plasma, and low in rabbit, mouse, and rat plasma. CES is only expressed at high levels in rabbit, mouse, and rat plasma, possibly in compensation for their low BChE levels. We also previously reported on the low catalytic ability of BChE compared with the extensive hydrolytic ability of CES (Bahar et al., 2012). In that study, we measured plasma hydrolytic activity using several substrates, and found that plasmas possessing a high level of BChE showed very low hydrolytic activity, in contrast to the high hydrolytic activity of plasmas with CES. We postulated that there may be an inverse relation between CES and BChE levels.
In mouse and rat plasma, various substrates are rapidly hydrolyzed by CES. However, rabbit plasma CES shows distinct substrate specificity and can hydrolyze only a limited number of substrates, such as butyrylthiocholine and propranolol derivatives (Bahar et al., 2012). Our current study showed aspirin to be another example of a substrate that is rapidly hydrolyzed by rabbit plasma CES, in contrast with rat and mouse plasma CESs. Although we cannot compare the sequence homology of these CESs due to lack of information on the alignment of rabbit plasma CES, mouse and rat plasma CES show 83% sequence homology (calculated by ClustalW alignment, rat CES1, NP_058700; mouse CES1, NP_0311980).

In the inhibition experiments, we observed an interesting phenomenon in that rat and mouse plasma CES activity for aspirin hydrolysis was enhanced by ethopropazine, especially rat plasma (4.6-fold; Table 1). Although the enhancing mechanism is unclear, there are two possibilities: a positive conformational change in the active center of CES and/or the appearance of nonspecific catalytic sites due to binding of ethopropazine. Because aspirin can also be hydrolyzed by nonspecific binding, as in the case of albumin, the binding of ethopropazine to CES may bring about exposure of nonspecific binding sites. No enhancing effect of ethopropazine was observed in rabbit plasma. The substrate specificities and enhancement of activity by ethopropazine confirmed the similarities of mouse and rat plasma CES and the dissimilarity of rabbit plasma CES.

Among the mammals without plasma CES, minipig plasma showed the lowest level of aspirin hydrolysis, nearly the same as that of 4% HSA. There have been some reports that the acetyl group of aspirin binds with the lysine residue of HSA and thus the acylation of HSA supports the hydrolyzing activity of human plasma (Morikawa et al., 1979; Yang et al., 2007; Liyasova et al., 2010). In addition to its activity with respect to albumin, aspirin has long been known to be a substrate for human plasma BChE (Hofstee, 1951; Morgan and Truitt, 1965; Morikawa et al., 1979; Valentino et al., 1981; Masson et al., 1998). In addition to BChE and albumin, it was recently reported that aspirin is hydrolyzed by plasma PON (Santanan and Parhasarathy, 2007; Jaichander et al., 2008). The results of the present study also indicate that the three proteins, PON, BChE, and albumin, are jointly responsible for aspirin hydrolysis in human, monkey, and dog plasma (Table 1). In human plasma, nearly 16%, 30%, and 50% of aspirin hydrolysis was due to albumin, BChE, and PON, respectively. During the hydrolysis experiments, the addition of calcium noticeably increased hydrolyase activities in human plasma and the plasma of some other animals (Fig. 1). These significant increases are not considered to be due to PON but rather to BChE (Table 2), in spite of the fact that PON requires calcium for substrate hydrolysis. Calcium did not affect the activities of rabbit or minipig hydrolases, since aspirin is primarily hydrolyzed by plasma CES in rabbit and is not hydrolyzed by any hydrolases in minipig plasma.

It is conceivable that the activation of hydrolyase activity in human, monkey, and dog plasma in the presence of calcium is related to their high BChE content. Interestingly, although dog plasma BChE plays only a minor role in hydrolyase activity, aspirin hydrolysis was still noticeably increased in the presence of calcium in this species. Even in species with a low BChE content, calcium stimulated the hydrolyase activity of this enzyme, since the effect of ethopropazine inhibition on mouse and rat plasma is significantly greater in the presence of calcium than in its absence (Tables 1 and 2). Sequence analysis revealed that human and monkey plasma BChEs show nearly 96% homology, whereas human and dog plasma BChEs show 87% (calculated by ClustalW alignment; human BChE, NP_000046; monkey BChE, ADD64703; dog BChE, XP_545267). ClustalW alignment analysis for BChE enzymes in mouse and rat plasma showed around 80% of sequence similarity to human BChE (mouse BChE, NP_033868; rat BChE, NP_075231). Here we report for the first time that calcium also has a stimulating effect on the plasma BChE of not only human, monkey, and dog but also mouse and rat.

In human, monkey, and dog plasma, the addition of magnesium also had a positive effect on hydrolyase activity by affecting plasma BChE (Fig. 2). To explain the positive effects of calcium and magnesium on BChE, Valentino et al. (1981) and Masson et al. (1998) have suggested a facilitating approach, in which negatively charged substrates (i.e., aspirin) form a complex with divalent cations and thereby enter more easily the active center of the BChE, which has a negative charge. However, another divalent cation, zinc, had a negative effect on hydrolyase activity (Fig. 2). Therefore, the positive effects of calcium and magnesium on BChE cannot be explained by complex formation between aspirin and divalent cations.

In an attempt to confirm the positive effect of calcium and magnesium on plasma BChE activity, their concentration dependency was studied in human plasma (Fig. 3). We found that BChE possesses high- and low-affinity binding sites for calcium and/or magnesium. A classic canonical consensus sequence was previously reported as a calcium binding site in an intervening loop between two helices (Kretsing et al., 1976). Schallreuter et al. (2007) found a single calcium binding site in each subunit of human plasma BChE. We found two binding sites for calcium in this study. The high-affinity binding site may be related to a canonical binding loop, whereas the second low-affinity binding site is observed for the first time in this study. Some researchers have performed activity analysis experiments with purified human plasma BChE using propionylthiocholine and/or butyrylthiocholine, and detected a possible allosteric increase in activity, produced by the addition of 1 mM calcium (Marquis, 1983). Other researchers have reported that 1 mM calcium and 1 mM magnesium do not affect the activity of BChE, but they help to recover its activity after inhibition by other divalent cations such as zinc, nickel, cobalt, and so forth (Sarkarati et al., 1999; Çokuğraş et al., 2003).

When calcium and/or magnesium are bound to BChE, it assumes a new conformation due to its allosteric properties. This form may be more appropriate for aspirin hydrolysis. It is known that, under physiologic conditions, free calcium and magnesium concentrations are 1.1–1.4 mM and 0.5–0.8 mM in plasma, respectively. However, in some disease states, the free calcium concentration in plasma increases (hypercalcemia), whereas concentrations over 1.8 mM suggest cancer, particularly carcinoma and leukemia. In contrast, magnesium concentrations are less changeable than plasma calcium levels as a result of disease, although in cases of renal failure they can increase up to 1.6 mM. The high binding affinities of calcium and magnesium (1.46 and 1.00 mM, respectively) are similar to their free concentrations under physiologic conditions.

There have been several reports claiming that different plasma proteins are the responsible enzymes for aspirin hydrolysis. This may be due to different experimental conditions (e.g., dilution of plasma by different buffers). Our results showed BChE to be the major responsible plasma protein for aspirin hydrolysis under physiologically normal concentrations of free calcium. However, we have no data on the number of bound divalent cations or whether this number is significant when considering the increase of hydrolyase activity of BChE. In addition, human BChE is known to be a soluble sugar-coated protein (23.9% by weight) consisting of nine N-linked carbohydrate chains (Lockridge et al., 1987; Li et al., 2008). We have no information as to whether any of these sugar chains are involved in the binding of divalent cations. We are planning to conduct further investigations to clarify this and to reveal the exact mechanism of the interaction between BChE and divalent cations.