Aspirin and Sodium Salicylate Inhibit Endothelin ETA Receptors by an Allosteric Type of Mechanism

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Received July 12, 1999; accepted December 28, 1999 This paper is available online at http://www.molpharm.org

ABSTRACT

Aspirin is a commonly used drug with a wide pharmacological spectrum including antiplatelet, anti-inflammatory, and neuroprotective actions. This study shows that aspirin and sodium salicylate, its major blood metabolite, reverse contractile actions of endothelin-1 (ET-1) in isolated rat aorta and human mammary arteries. They also prevent the intracellular Ca\(^{2+}\) mobilizing action of ET-1 in cultured endothelial cells but not those of neuromedin B or UTP. Inhibition of the actions of ET-1 by salicylates is apparently competitive. Salicylates inhibit \(^{125}\)I-ET-1 binding to recombinant rat ETA receptors. Salicylic acid promotes dissociation of \(^{125}\)I-ET-1 ETA receptor complexes both in the absence and the presence of unlabeled ET-1. It has no influence on the rate of association of \(^{125}\)I-ET-1 to ETA receptors. Salicylates do not promote dissociation of \(^{125}\)I-ET-1 ETB receptor complexes. Salicylates potentiate relaxing actions of receptor antagonists such as bosentan. It is concluded that salicylates are allosteric inhibitors of ETA receptors. The results also suggest that: 1) irreversible ET-1 binding probably limits actions of receptor antagonists in vivo, and 2) an association of salicylates and ETA receptor antagonists should be used to evaluate the physiopathological role of ET-1 and may be of therapeutic interest in the treatment of ischemic heart disease.

Aspirin (acetylsalicylic acid) has antiplatelet, anti-inflammatory, and neuroprotective actions, but its molecular mechanisms of action are complex. Aspirin was reported two decades ago to inhibit platelet cyclooxygenase activity and the formation of thromboxanes (Patrono, 1994). A growing number of studies suggest, however, that anti-inflammatory actions of aspirin may not be related to the inhibition of prostaglandin synthesis. For instance, salicylates activate p38 mitogen-activated protein kinase and inhibit tumor necrosis factor-induced IKB phosphorylation (Schwenger et al., 1997, 1998).

A major clinical use of aspirin therapy is to improve the survival of patients with myocardial infarction and unstable angina (ISIS-2, 1988). This action is usually attributed to the antiplatelet action of aspirin, but participation of other mechanisms of action cannot be excluded. For instance, aspirin has recently been shown to protect endothelial cells against oxidative stresses, possibly by promoting the synthesis of ferritin (Oberle et al., 1998).

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide (Yanagisawa et al., 1988) that plays an important role in several diseases thought to be associated with vasoconstrictions. These are coronary vasospasm (Toyo-oka et al., 1991), unstable angina (Weiczorek et al., 1994), myocardial infarction (Omland et al., 1994), cardiac insufficiency (Mulder et al., 1997), and cerebral vasospasm associated with subarachnoid hemorrhage (Clozel et al., 1993). The fact that protective actions of aspirin are observed in conditions that are associated to increased circulating levels of ET-1 suggested to us that aspirin may have anti-ET-1 properties. This study was designed to test this hypothesis. It shows that salicylates are allosteric inhibitors of ETA receptors.

Materials and Methods

Chemicals. ET-1, indo-1/AM, indomethacin, neuromedin B, UTP, aspirin, and sodium salicylate (SA) were obtained from Sigma Chemical Co. (St. Louis, MO). \(^{125}\)I-ET-1 (2200 Ci/mmol) was prepared as described previously (Desmarets et al., 1996). Bosentan was provided by Dr. M. Clozel (Actelion, Basel, Switzerland). All effectors were dissolved in an Earle’s salt solution. Composition of the solution was: 140 mM NaCl, 5 mM KCl, 0.8 mM MgSO\(_4\), 1.8 mM CaCl\(_2\), 25 mM HEPES, pH 7.4.

Cell Cultures. Rat brain capillary endothelial cells of the B7 clone were grown as described previously (Vigne et al., 1995). Stable transfectant CC39 cells expressing functional rat ETA or rat ETB receptors were maintained as described.

ABBREVIATIONS: ET-1, endothelin-1; SA, sodium salicylate.

This work was supported by the Centre National de la Recherche Scientifique and the Fondation de France.
receptors were prepared as described previously (Gresser et al., 1996). Cell homogenates were prepared as described previously (Desmaret et al., 1996) and stored at −20°C. Proteins were determined according to Bradford (1976) using BSA as standard. Receptor densities were 0.4 ± 0.1 pmol/mg of proteins in membranes prepared from B7 cells (Vigne et al., 1990). They were 26 ± 1 and 4 ± 0.5 pmol/mg in membranes prepared from ETA and ETB receptor-expressing fibroblasts, respectively.

**Contraction Experiments.** Thoracic aorta from 200-g female Wistar rats were cleaned of adherent fat, cut into rings, and the endothelium was removed. Human internal mammary arteries from seven patients (six men and one woman, age 56–83, mean 63.5 ± 4.3 years) undergoing bypass surgery were collected. All the procedures followed were in accordance with institutional guidelines. In the operating room, fragments were immediately placed in a Krebs’ solution buffered at pH 7.4 with 25 mM HEPES/NaOH. Fragments were cleaned of adherent connective tissues. The endothelium was removed by gently rubbing the intimal surface with the tips of small forceps and 3- to 4-mm wide rings were prepared.

Rings were mounted under 2 g resting tension in organ baths (3 ml, 37°C, bubbled with a 5% CO₂ and 95% O₂ gas mixture) containing Krebs bicarbonate solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 2 mM CaCl₂, and 5.8 mM glucose; pH 7.4. After a 60-min period of equilibration during which the buffer was changed at 15-min intervals, four contraction/relaxation cycles were performed on a Gould TA4000 recorder.

**Data Presentation and Statistical Analysis.** Data are given as mean ± S.E. and the number of experiments performed. Statistical analysis was performed with the paired t test. Values of P < .05 were considered statistically significant. Binding data were analyzed using the Ligand software (Jandel Scientific, Corte Madera, CA). When no error bar is present on a figure, it was smaller than the size of the points.

**Results**

**Anti-ET-1 Actions of Salicylates on Rat Aortic Rings.** ET-1 induced large and irreversible contractions of isolated rat aortic rings. These contractions are mediated by ETA receptors (Marsault et al., 1991). Figure 1 presents typical recordings that show that addition of aspirin (Fig. 1A) or SA (Fig. 1B) to arteries that had been precontracted with ET-1 induced rapid and partial relaxations. Data obtained are summarized in Fig. 1, C and D. Figure 1C shows that the half-time of relaxation induced by aspirin was 3.4 ± 0.4 min (n = 21). Figure 1D shows a dose-dependent increase in the maximum extent of the relaxations induced both by aspirin and SA.

Cumulative dose-response curves for the action of ET-1 were established in the absence and the presence of SA. Figure 2 shows that SA shifted the dose-response curves for the actions of ET-1 to larger concentrations. These suggested a competitive type of inhibition. Indomethacin (10 μM) did not alter the ET-1 dose-response curve. Identical relaxing actions of salicylates were observed in intact aortic rings and in endothelium-denuded rings (not shown).

Relaxing actions of salicylates were different from those of bosentan, an endothelin receptor antagonist (Clozel et al., 1993). Application of bosentan to arteries that had been precontracted with 100 nM ET-1 induced slow and almost complete relaxations. Maximum relaxations observed after 90 min of exposure to 10 μM bosentan were 90 ± 3% (n = 5). The half-time of relaxations induced by bosentan was 38 ± 4 min (n = 5). It was 10 times longer than that observed for salicylates.

**Anti-ET-1 Actions of Salicylates on Human Mammary Arteries.** Similar experiments were performed using rings of human internal mammary arteries. This preparation has already been reported to respond to ET-1 by large and sustained contractions that are mainly due to ETA receptors (Seo et al., 1994; Maguire and Davenport, 1995). Experiments were performed using endothelium-denuded arteries and in the presence of 10 μM indomethacin to prevent a possible action of aspirin on cyclooxygenase. Figure 3 shows that SA and aspirin relaxed arteries that had been precontracted with ET-1. The time courses of the relaxations were very similar to those observed with rat aortic rings. Figure 3C also shows cumulative dose-response curves for the ac-
tions of ET-1 in the absence or presence of SA. SA inhibited actions of all concentrations of ET-1 > 1 nM.

**Salicylates Prevented ETA Receptor-Mediated Intracellular Ca\(^{2+}\) Mobilization.** We next looked for antie-1 actions of salicylates at a cellular level. Endothelial cells from brain capillaries were chosen for different reasons: 1) unlike endothelial cells from large peripheral vessels, they express ETA receptors (Vigne et al., 1990); 2) they express two other phospholipase C-coupled heptahelical receptors: neumedin B-prefering bombesin receptors (Vigne et al., 1995) and P2Y2 purinergic receptors (Frelin et al., 1993); and 3) pharmacological properties of receptors can be conveniently monitored by flow cytometric analyses of indo-1-loaded cells (Vigne et al., 1990). Figure 4A shows that SA and aspirin decreased the ET-1 induced [Ca\(^{2+}\)]\(_i\) rise in indo-1-loaded cells. Half maximum inhibition was observed at 2 mM SA and 3 mM aspirin.

The dose-response curve for ET-1 induced [Ca\(^{2+}\)]\(_i\) rise is shown in Fig. 4B. Half maximum increase in [Ca\(^{2+}\)]\(_i\) was observed at 20 nM ET-1. SA shifted the ET-1 dose-response curve to larger concentrations. Application of the Cheng-Prusof relationship indicated an apparent \(K_i\) value for SA of 7 mM.

A concentration of SA of 10 mM that inhibited 80% of ET-1 induced [Ca\(^{2+}\)]\(_i\) increases did not modify the Ca\(^{2+}\) mobilizing actions of 1 \(\mu\)M neumedin B that were mediated by neumedin B preferring bombesin receptors. It did not modify the Ca\(^{2+}\) mobilizing actions of 10 \(\mu\)M UTP that were mediated by P2Y2 purinergic receptors. These indicated that actions of SA were specific for ETA receptor. These results also ruled out a possible nonspecific action of SA on the Ca\(^{2+}\) signaling cascade.

**Salicylates Inhibited ET-1 Binding to Recombinant ETA Receptors.** To further analyze the mechanism of action of salicylates, we performed binding experiments using \(^{125}\)I-ET-1 and recombinant rat ETA receptors. Figure 5 shows that salicylates prevented \(^{125}\)I-ET-1 binding. Half maximum inhibition was observed at 10 mM aspirin or SA. Bosentan was much more potent.

**Salicylates Modified the Properties of Interaction of ET-1 with ETA Receptors.** We next analyzed the kinetics of association of \(^{125}\)I-ET-1 to ETA receptors and the kinetics of dissociation of the complexes in the presence of different concentrations of SA. Results are shown in Fig. 6. Association or \(^{125}\)I-ET-1 to ETA receptors in the absence of SA did not follow pseudo first order kinetics for reasons that have been described previously (Desmarets et al., 1996). We observed, however, that association kinetics in the presence of SA could be linearized according to pseudo first order processes. Table 1 shows that increasing doses of SA did not modify the second order rate constant of association of \(^{125}\)I-ET-1 to its receptors. Dissociation kinetics in the absence or the presence of SA were monoeaxponential. The half-life of \(^{125}\)I-ET-1 ETA receptor complexes was 12.4 ± 0.5 h \((n = 11)\) in the absence of SA, meaning an almost irreversible binding. SA increased up to 10-fold the rate constant of dissociation of \(^{125}\)I-ET-1 receptor complexes (Table 1). The apparent equilibrium dissociation constant \((K_d)\) of ET-1 ETA receptor complexes was estimated to be 1.4 pM in the absence of SA. It increased to 12 pM in the presence of 50 mM SA. A Schild plot of the data indicated an apparent \(K_i\) value for SA of 8 mM. Thus SA decreased the apparent affinity of ET-1 for ETA receptors by favoring dissociation of ET-1 receptor complexes.

In a second series of experiments, \(^{125}\)I-ET-1 ETA receptor complexes were allowed to form in the absence of SA. Figure 7 shows that addition of SA, in the absence of unlabeled ET-1, induced a partial dissociation of the complexes. Increasing doses of SA increased the fraction of dissociable sites in a manner that was strikingly similar to the pattern of SA-induced relaxations (Fig. 1).

To further ascertain that the apparent decreases in affinity of ET-1 for its receptors induced by SA quantitatively accounted for the lower \(^{125}\)I-ET-1 bindings observed in Figs. 6A and 7 we used the \(K_d\) values listed in Table 1 to calculate the expected effect of SA on equilibrium \(^{125}\)I-ET-1 binding. Table 2 shows that there was a close agreement between expected and observed decreases in \(^{125}\)I-ET-1 binding. These results indicated that SA did not act as a simple competitive antag-
onist of ETA receptors. It is an allosteric inhibitor of ETA receptors.

$^{125}$I-ET-1 and ETB receptors also form tight complexes that hardly dissociate. Figure 8 shows that large concentrations of aspirin and SA (50 mM) that accelerated 10 times the dissociation of $^{125}$I-ET-1 ETA receptor complexes (Table 1), did not induce dissociation of $^{125}$I-ET-1 ETB receptor complexes. Thus, allosteric actions of salicylates are specific for ETA receptors.

We also checked that acetic acid and Na benzoate, which are the component parts of aspirin and SA, at a concentration of 30 mM had no action on the dissociation of $^{125}$I-ET-1 ETA receptor complexes (data not shown). Finally we checked that SA (20 mM) did not induce a degradation of free or receptor-bound $^{125}$I-ET-1.

Salicylates Potentiated Actions of Receptor Antagonists. The major interest of an allosteric inhibitor of ETA receptors is that it transforms an almost irreversible ET-1 binding into a reversible process. One consequence is that salicylates should favor replacement of bound ET-1 by receptor antagonist and thus potentiate actions of receptor antagonists. This hypothesis was tested using binding experiments and contraction experiments.

$^{125}$I-ET-1 ETA receptor complexes were allowed to form in the absence of salicylates. Addition of unlabeled ET-1 induced a decrease in the bound radioactivity, which indicated that $^{125}$I-ET-1 dissociated slowly from the receptors and was replaced by unlabeled ET-1. This type of experiment is in fact used to analyze dissociation kinetics of receptor ligand complexes. Figure 9A shows that addition of 20 mM SA induced a much faster replacement of bound $^{125}$I-ET-1 by unlabeled ET-1. Aspirin was as potent as SA. We next used bosentan instead of unlabeled ET-1. Figure 9B shows that addition of $10 \times 10^{-6} M$ bosentan to $^{125}$I-ET-1 receptor complexes induced a small decrease of the bound radioactivity. The half-life of $^{125}$I-ET-1 receptor complexes determined in the presence of bosentan was $11.4 \pm 1.0 \text{ h} (n = 3)$. Identical values were obtained in experiments using unlabeled ET-1 (12.4 $\pm$ 0.5 h) or mixtures of $100 \times 10^{-9} M$ ET-1 and $10 \times 10^{-6} M$ bosentan ($10.6 \pm 0.9 \text{ h}, n = 3$). These indicated that bosentan is not an allosteric inhibitor of ETA receptors. Finally, Fig. 9B shows that SA (20 mM) accelerated the dissociation of $^{125}$I-ET-1 receptor complexes in the presence of bosentan, meaning that SA

![Fig. 2. Cumulative ET-1 dose-response curves in the absence or the presence of 10 mM SA (A) or 30 mM SA (B). Two aortic rings from the same rat were mounted in two parallel organ chambers. One ring served as a control. The other was treated with 10 or 30 mM SA. The two rings were then exposed to increasing doses of ET-1 (1, 3, 10, 30, and 100 nM) and changes in tension were recorded. Mean ± S.E. (n = 12 in A, n = 4 in B) are indicated. **P < .01, *P < .05 using a paired t test. Tensions were normalized to that produced by the last KCl contraction.](image)

![Fig. 3. Anti-ET-1 actions of salicylates in isolated human mammary arteries. A, representative traces showing relaxing actions of 30 mM SA and 30 mM aspirin on endothelium-denuded rings of mammary arteries. Horizontal bar: 2 min, vertical bar: 0.6 g. C, cumulative ET-1 dose-response curves in the absence (○) or the presence (●) of 10 mM SA. Experiments were performed as described in the legend of Fig. 2. Mean ± S.E. (n = 5) are indicated. **P < .01, *P < .05 using a paired t test. Tensions were expressed relative to that observed in response to 100 nM ET-1 in the control ring.](image)

![Fig. 4. SA specifically inhibited ET-1 induced intracellular Ca$^{2+}$ mobilization in brain capillary endothelial cells. Left: dose-response curve for the inhibition by SA (○) and aspirin (●) of ET-1 (100 nM)-induced intracellular Ca$^{2+}$ mobilization. Right: dose-response curves for the Ca$^{2+}$ mobilizing action of ET-1. Experiments were performed in the absence (○) or the presence (●) of 20 mM SA. Mean ± S.E. (n = 3) are indicated.](image)

![Fig. 5. Inhibition by salicylates of $^{125}$I-ET-1 binding to recombinant ETA receptors. $^{125}$I-ET-1 binding to ETA receptors was measured in the presence of increasing doses of aspirin (○), SA (●), and bosentan (■). The concentration of $^{125}$I-ET-1 was 10 pM.](image)
facilitated replacement of bound ET-1 by bosentan. It is of
interest to note that less than 10% of \(^{125}\text{I}\)-ET-1 receptor
complexes dissociated after a 4-h exposure to bosentan.
About 70% of the complexes dissociated during the same time
in the presence of bosentan and SA.

We next tested whether SA promoted the relaxing action of
bosentan. SA (10 mM) by itself induced a 38 ± 3% (n = 9)
relaxation of ET-1 (100 nM)-contracted aortic rings. Bosen-
tan (10 µM) by itself induced a 90 ± 3% relaxation after 90
min. Figure 10A shows that addition of 10 mM SA after
bosentan induced a rapid and almost complete relaxation of
the arteries. Conversely, addition of bosentan to arteries that
had been partially relaxed by 10 mM SA induced fast and
complete relaxations (Fig. 10B). The half-time for the relax-
ations induced by bosentan was 12.2 ± 0.7 min (n = 13). It
was 3 times less than the corresponding time observed in the
absence of SA (38 ± 4 min). Thus SA accelerated relaxing
actions of bosentan 3-fold.

**Fig. 6.** Association and dissociation kinetics of \(^{125}\text{I}\)-ET-1 to ETA recep-
tors. A, association of \(^{125}\text{I}\)-ET-1 (4 pM) to ETA receptors was followed in
the absence (○) or the presence of 20 (●), 30 (▲), or 50 mM (■) SA. B, after
association of \(^{125}\text{I}\)-ET-1 to ETA receptors in the absence (○) or the
presence of 20 (●) or 30 mM SA (▲), dissociation kinetics were initiated by the
addition of 100 nM unlabeled ET-1 to the tubes.

**Discussion**

**Why Is It Important to Relieve Irreversible ET-1
Binding?** Quasi-irreversible ET-1 binding was recognized
very early but its consequences are not yet fully understood.
Waggoner et al. (1992) first described that the slow rate of
dissociation of ET-1 receptor complexes implies \(K_d\) values
of ET-1 receptor complexes in the pM range. We also showed
that equilibrium binding studies can greatly underestimate
affinity constants for both ET-1 and competing ligands un-
less special care is not taken to avoid time-limited second
order kinetic conditions (Desmarets et al., 1996). Irreversible
binding is responsible for atypical pharmacological responses
such as the lack of action of guanine nucleotides (Nambi et
al., 1996) and actions of receptor antagonists (Leite et al.,
1994). It is responsible for long-lasting actions of ET-1 and
the development of long-term refractoriness. Recovery from
refractoriness is variable from tissue to tissue. It is very slow
in the cardiac muscle (Leite et al., 1994; Hilal-Dandan et al.,
1997) but faster in the rat aorta (Marsault et al., 1993) and
probably limited by the rate of externalization of newly
formed or recycling receptors (Marsault et al., 1993). Quasi-
irreversible binding may account for the fact that functional
ET receptors serve as clearance receptors (Frelin and Gué-
din, 1994). It imposes conditions in which autocrine actions
(that is, vasodilating) of ET-1 should be favored over para-
crine actions (that is, vasoconstricting) as observed in ET-1
induced vasoconstrictions (Frelin and Guédin, 1994). Finally,
irreversible binding implies that ET-1 is more likely to contrib-
ute to long-term physiological or pathophysiological regula-
tions than to short-term regulations (Hilal-Dandan et al.,
1997). Relieving the irreversibility of ET-1 binding is there-
fore important for a better understanding of the role of ET-1.
It can be achieved using allosteric inhibitors of the receptors.
This study shows that aspirin and SA are allosteric inhibitors
of ETA receptors.

**Salicylates Are Allosteric Modifiers of ETA Recep-
tors.** Salicylates reverse ET-1-induced contractions in iso-
lated rat aortic rings and human mammary artery rings
(Figs. 1 and 3). These actions were observed in the absence of
endothelium and for human tissues, in the presence of indom-
ethacin. This, together with the observation (in the differ-
ent assays that have been performed) that SA is slightly
more potent than aspirin suggests that actions of salicylates
are unlikely to be mediated by an inhibition of cyclooxygen-
ase.

**TABLE 1**

Kinetic parameters of \(^{125}\text{I}\)-ET-1 binding to rat ETA receptors

<table>
<thead>
<tr>
<th>Conditions</th>
<th>(k_a) ((M^{-1}\text{min}^{-1}))</th>
<th>(k_d) (min(^{-1}))</th>
<th>Half-time (h)</th>
<th>(K_d) (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>9.3 (10^{-4})</td>
<td>12.4 ± 0.5 (n = 11)</td>
<td>1.4 ± 0.3†</td>
</tr>
<tr>
<td>10 mM SA</td>
<td>6.4 (10^8)</td>
<td>1.7 (10^{-3})</td>
<td>6.6</td>
<td>2.7</td>
</tr>
<tr>
<td>20 mM SA</td>
<td>6.6 (10^8)</td>
<td>2.7 (10^{-3})</td>
<td>4.3 ± 0.5 (n = 7)</td>
<td>4.1 ± 0.4</td>
</tr>
<tr>
<td>30 mM SA</td>
<td>6.6 (10^8)</td>
<td>4.8 (10^{-3})</td>
<td>2.4 ± 0.2 (n = 2)</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>50 mM SA</td>
<td>7.6 (10^8)</td>
<td>8.9 (10^{-3})</td>
<td>1.3</td>
<td>11.7</td>
</tr>
</tbody>
</table>

ND, Not determined for association kinetics in the absence of SA did not follow pseudo first order kinetics.

† This value was calculated by assuming a \(k_a\) value of 6.3 \(10^8\) M\(^{-1}\)min\(^{-1}\). This value is the mean of the four values determined in the presence of SA.
Salicylates have anti-ET-1 actions in isolated vessels but also in isolated cells. They prevent the Ca$^{2+}$ mobilizing action of ET-1 in isolated endothelial cells but not those of neuromedin B or UTP (Fig. 4). In the two preparations, salicylates shift the dose-response curves for the actions of ET-1 to larger concentrations, which may be suggestive of a competitive type of inhibition. This conclusion is supported by the results of binding experiments that show that SA prevents $^{125}$I-ET-1 binding to recombinant ETA receptors by decreasing the apparent affinity of the receptors for ET-1 (Table 1). The mechanism of action of SA is more complex, however, for it promotes dissociation of $^{125}$I-ET-1 receptor complexes (Figs. 6 and 7). These observations are strong indications that salicylates do not bind to the ET-1 site on ETA receptors and allosterically modify the interaction of ET-1 with its receptors. Whether salicylates act on the receptor itself, or on an associated protein, cannot be defined from our experiments.

Allosteric modulation of G protein-coupled receptors has already been described. It is best characterized with muscarinic receptors (Tucek and Proska, 1995) and has been studied to a limited extent with other receptors such as adenosine A1, α2-adrenergic, and D2 dopamine receptors (Bruns and Fergus, 1990; Hoare and Strange, 1998; Leppik et al., 1998). It was not described previously for ETA receptors. Allosteric modifiers of G protein-coupled receptors described so far are not structurally related to salicylates. Amiloride derivatives (benzamil and ethylisopropyl amiloride) that modify D2 dopamine receptors (Hoare and Strange, 1998) and α2-adrenergic receptors (Nunnari et al., 1987; Leppik et al., 1998) are inactive on ETA receptors (data not shown). We also show that concentrations of SA that inhibit ET-1 responses have no influence on neuromedin B and UTP responses in endothelial cells (Fig. 4). These responses are mediated by neuromedin B-prefering bombesin receptors (Vigne et al., 1995) and P2Y2 receptors (Frelin et al., 1993), respectively. In addition, salicylates do not induce dissociation of ET-1 ETB receptor complexes (Fig. 8). Thus, allosteric actions of salicylates are probably specific for ETA receptors.

### Specificity of the Action of Salicylates

Actions of salicylates reported here were observed at large millimolar concentrations of the drugs. At such concentrations, weak...
organic acids and salicylates are known to alter membrane fluidity (Watala and Gwozdzinski, 1993; Balasubramanian et al., 1997) and to act as mild chautrophic agents. The following evidence suggests that actions of salicylates described here are not nonspecific: 1) SA selectively modifies the rate of dissociation of ET-1 ETA receptor complexes; 2) SA does not alter ETB receptor, neuremodin B-prefering bombesin receptors, and P2Y2 purinergic receptors; 3) Na benzoate did not induce dissociation of ET-1 receptor complexes; and 4) SA promotes dissociation of solubilized ET-1 receptor complexes (data not shown). The possibility that SA induced a degradation of free or receptor-bound ET-1 was also ruled out.

**Relaxing Actions of Salicylates and Bosentan.** The proposed mechanism of action of SA and aspirin can account (at least qualitatively) for the results obtained in functional assays. These are: 1) that fact that salicylates produced faster relaxations than bosentan, and 2) apparent competitive antagonism observed using intact vessels (Figs. 2 and 3) and endothelial cells (Fig. 4).

It is also of interest to note that although salicylate-induced relaxations (Figs. 1 and 3) closely resemble salicylate-induced dissociations of $^{125}$I-ET-1 receptor complexes (Fig. 7), their time courses differ. Relaxations induced by salicylates are faster than salicylate-induced dissociation of ET-1 receptor complexes. A similar difference is observed with bosentan. The half-time for bosentan-induced relaxations (38 min) is much shorter than the half-life of ET-1 receptor complexes measured in the presence of bosentan (11.4 h). In other words, relaxations induced by bosentan or salicylates are observed under conditions in which most ET-1 remains bound onto receptors in experiments using membranes. One reason for this discrepancy could be that only receptors that recycle to the plasma membrane contribute to steady-state tension of isolated aortic strips (Marsault et al., 1991). Receptor antagonists that do not induce dissociation of ET-1 receptor complexes relax arteries, for they compete with ET-1 for the occupancy of receptors (newly synthesized or recycling) that externalized to the plasma membrane. Under these conditions, the half-time for relaxations induced by receptor antagonists is not a measure of the half-time of ET-1 receptor complexes. It is a measure of the rate at which new receptors externalize to the plasma membrane (Marsault et al., 1991). The same mechanism may account for salicylate-induced relaxations. One difference is, however, that although receptor antagonist can only compete with ET-1 for the occupancy of free receptors and cannot act if ET-1 has already bound to receptors, salicylates both prevent ET-1 binding and dissociate preformed complexes. This may be a reason why the half-time for salicylate-induced relaxations is faster than the half-time for bosentan-induced relaxations.

**Pharmacological Implications.** The most important observation of this study is that SA potentiates relaxing actions of bosentan (Fig. 10). Several potent antagonists of ET receptors have been devised (Cheng et al., 1994). They are usually potent in protocols in which animals are treated at the same time with ET-1 and with receptor antagonists, i.e., under conditions in which antagonists and ET-1 compete for the same free receptors. Antagonists are usually less potent when administered to prevent actions of endogenous, receptor-bound, ET-1. One alleged reason is that ET-1 plays a minor role in the physiopathological conditions that have been assessed. The observation that SA potentiates relaxing actions of bosentan (Fig. 10) suggests that actions of receptor antagonists may be limited by the slow rate of dissociation of ET-1 receptor complexes. Indeed, a competitive antagonist can only act if receptors are free from endogenous ET-1. Our results thus suggest that an association of salicylates and ETA receptor antagonists could be useful to evaluate the physiopathological role of ET-1 and may be of therapeutic interest is the treatment of ischemic heart disease.

**Clinical Implications.** Aspirin has clear beneficial actions in coronary heart disease. Recommended daily doses of aspirin for the prevention of myocardial infarction and vascular death in patients with coronary heart disease are about 200 mg. Larger doses may be needed in patients with cerebrovascular diseases (Patrono and Roth, 1996). This study shows that millimolar concentrations of SA are needed to inhibit contractile actions of ET-1. Similar plasma concentrations of SA are only obtained with high anti-inflammatory doses of aspirin. This indicates that the mechanisms described in this article are unlikely to contribute to the beneficial actions of antiplatelet doses of aspirin in coronary heart disease.

**Acknowledgments**

We thank Prof. J. Jordan for providing us with mammary arteries, and J. Kervella and N. Boyer for technical assistance.

**References**


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