Capsazepine, a vanilloid antagonist, abolishes tonic responses induced by 20-HETE on guinea pig airway smooth muscle

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Capsazepine, a vanilloid antagonist, abolishes tonic responses induced by 20-HETE on guinea pig airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 288: L460–L470, 2005. First published November 19, 2004; doi:10.1152/ajplung.00252.2004.—The aim of this study was to delineate the mode of action of 20-hydroxy-eicosatetraenoic acid (20-HETE) in airway smooth muscle (ASM) cells. ASM metabolizes arachidonic acid by various enzymatic pathways, including the cytochrome P-450 (CYP-450) ω-hydroxylase, which leads to the production of 20-HETE, a bronchoconstrictive eicosanoid. The present study demonstrated that 20-HETE induced concentration-dependent tonic responses in ASM, whereas transient responses were recorded in Ca²⁺-free solution, suggesting an intracellular Ca²⁺ release process. 20-HETE inotopic responses were abolished by 36 μM 2-aminoethoxydiphenyl borate or 1 μM thapsigargin but were insensitive to 10 μM ryanodine, indicating that inositol triphosphate receptors likely control the release of intracellular Ca²⁺. Sustained tension, which required Ca²⁺ entry, was partially blocked by 1 μM nifedipine (an L-type) and 100 μM Gd³⁺ (a nonselective cationic channel blocker). Moreover, in the absence of selective 20-HETE receptor antagonists, 20-HETE tonic responses were inhibited in a concentration-dependent manner (0.1–10 μM) by capsazepine, a well-characterized vanilloid receptor antagonist. Capsazepine was also observed to reverse cumulative responses to 20-HETE and capsaicin, a TRPV1 agonist. In addition, capsazepine pretreatment largely modified the sustained inotropic responses to 20-HETE, suggesting that 20-HETE cross-reacted with TRPV1 receptors with a low affinity (μM) or that its specific receptor was inhibited by the vanilloid antagonist. Data obtained using RH-80267, ONO-218-082, and eicosatetraynoic acid, respective inhibitors of diacylglycerol-lipase, phospholipase A₂, and CYP-450 ω-hydroxylase, reveal that intracellular arachidonic acid production and its metabolite may be responsible for the activation of nonselective cationic channels and tonic responses.

20-hydroxyeicosatetraenoic acid; contraction; lung; TRPV channel

RECENT REPORTS HAVE DEMONSTRATED that 20-hydroxyeicosatetraenoic acid (20-HETE), a bioactive arachidonic acid (AA) metabolite produced by cytochrome P-450 (CYP-450) ω-hydroxylase, is an important modulator of vascular, kidney, gastrointestinal, and bronchial cell reactivity (for review see Ref. 25). Moreover, its production may be affected in vivo under certain pathological conditions (25). Exogenous addition of 20-HETE at submicromolar concentrations was also shown to increase smooth muscle tone in guinea pig airway smooth muscle (ASM), an effect largely due to specific electrophysiological processes such as direct activation of various surface membrane ionic conductances (8) including the recently demonstrated nonselective cationic channels such as transient receptor potential type C6 (TRPC6) (2). Opposite mechanical effects, however, have also been reported in vivo in ASM from various species, including rabbit and humans (13, 33). One of the challenging issues in the present field is that, to our knowledge, there are presently no specifically identified or cloned 20-HETE receptors. This issue also applies for epoxyeicosatrienoic acid (EET) regioisomers, which, by contrast, are well-characterized hyperpolarizing and relaxing agents and described as large conductance calcium-activated potassium activators in several studies on either vascular smooth muscle (1, 12) or ASM (4, 32). It has been postulated that “orphan agonists,” such as EET and 20-HETE, may act through a subfamily of G protein-coupled receptors (GPCR), although evidence is mostly indirect (10). On the other hand, there are increasing evidence and direct demonstrations that 20-HETE is specifically active in smooth muscle cells (4, 24). Despite multiple attempts, however (9, 13), little is known as to the precise mechanisms by which this eicosanoid triggers and sustains tension increases in ASM tissues. This may be related to the absence of specific antagonist or to limited selectivity and/or availability of previously described inhibitors of 20-HETE synthesizing enzyme (13, 19). The fact that this AA cascade-derived eicosanoid displays bronchoconstrictive properties in the guinea pig has prompted further investigation into better defining 20-HETE signaling pathways. Although assessment of an eventual mobilization of an intracellular Ca²⁺ pool is rather straightforward, it still remains to be ascertained by experimental data. On the other hand, the activation of TRP channels in smooth muscle cells has already received close scrutiny (2, 8, 30). Taking advantage of a heterologous overexpression system, investigators have demonstrated the role of nonselective cationic conductances, such as TRPC6, in supporting tonic responses at both the cellular and membrane levels (2). However, from a functional and electrophysiological standpoint, Ca²⁺ permeability through TRPC channels has been reported to be quite low (5, 14), raising the possibility that 20-HETE could cross-activate other members of the TRP channel superfamily, whose lineage and basic properties have already been reviewed (6, 7). This family of transmembrane protein includes the canonical TRPC1–7 channels, the melastatin receptors, defined as TRPM1–4, and the TRPV1–4 subfamily and from which cDNA sequences are closely related to the first identified member and cloned as the vanilloid receptor TRPV1 (7, 29). Among these TRP-related proteins, TRPV1, V4, V5, and TRPV6 are the most permeable to Ca²⁺ (7, 22). Moreover, a number of studies have previously reported that
some members of the TRPC subfamily can be activated in a store depletion-independent manner by products of the phospholipase C (PLC) signaling cascade. Indeed, diacylglycerol (DAG)-dependent activation has been demonstrated for TRPC3/6/7 (11, 21), as well as for TRPC1/TRPC3 heteromers (28). In fact, DAG-lipase, which hydrolyzes the polyunsaturated fatty acid in the sn2 position, is quite active in most tissues and may ultimately release AA and yield to formation of metabolites. Among these, 20-HETE, the hydroxy-AA derivative, was shown to increase tone in vascular (25) and ASM cells (8). Moreover, these isotropic effects were shown to occur through the activation of intracellular Ca\(^{2+}\) release and TRPC-related conductances (8). These observations are of putative clinical interest for asthmatic and chronic obstructive pulmonary disease patients since the role of ASM cells is often raised in these pathophysiological conditions. Moreover, it has been reported that 20-HETE is synthesized in response to epidermal growth factor and may in fact be a mitogen, which suggests possible involvement in tissue remodeling (17).

The primary aim of the present work was to verify whether a specific Ca\(^{2+}\) pool is in fact mobilized by exogenous addition of 20-HETE in a guinea pig model. The second objective was to test whether the isotropic effects induced by this eicosanoid on native bronchi could be antagonized by known blockers and inhibitors of physiological and pharmacological interest. For this purpose, we assessed the mechanical and electrophysiological effects of 20-HETE in ASM at both tissular and cellular levels by examining the isotropic effects of 20-HETE using different pharmacological tools and experimental conditions. We were able to demonstrate that 1) 20-HETE activates the inositol (1,4,5)-triphosphate (InsP\(_3\)) cascade, which leads to InsP\(_3\) receptor activation, and 2) 20-HETE activates nonselective conductances that may facilitate a delayed calcium entry, which in turn could control tonic response. These results clearly provide new evidence that this process is sensitive to a specific vanilloid antagonist and enzymatic inhibitors and shed new light on the complex mode of action of eicosanoids, such as 20-HETE.

**MATERIALS AND METHODS**

**Isometric tension measurements.** The mechanical effects of 20-HETE were measured on helically cut tracheae and main bronchi excised from male and female albino guinea pigs (Hartley, 250–300 g) and dissected as previously reported (8). A Krebs solution containing (in mM) 118.1 NaCl, 4.7 KCl, 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 2.5 CaCl\(_2\), 2 H\(_2\)O, and 11.1 glucose, pH 7.4 upon carbogen bubbling 95% O\(_2\), 5% CO\(_2\), was used as the main physiological solution. 2-Aminoethoxydiphenyl borate (2-APB), BSA, carbacol, capsaicin, capsazepine, collagenase type IV, elastase type IV and nifedipine were purchased from Sigma (Oakville, ON, Canada). Gadolinium chloride (GdCl\(_3\)) was purchased from ICN Biomedicals (Cleveland, OH). The enzymatic inhibitors 1,6-bis-(cyclohexylloximinocarbonylaminol)-hexane [RH-80267 (U-57908)] and 2-(p-amlycinnamoyl) amino-4-chlorobenzoic acid (ONO-RS-082) were purchased from Biomol (Plymouth Meeting, PA). FBS, penicillin-streptomycin, and all cell media were purchased from GIBCO Invitrogen (Burlington, ON, Canada).

**Data analysis and statistics.** Results are expressed as means ± SE with n representing the number of experiments. Statistical analyses were performed using either paired or unpaired Student’s t-tests and analysis of variance testing. Values of P < 0.05 were considered to be statistically significant.

**RESULTS**

**Mobilization of a specific intracellular Ca\(^{2+}\) pool upon 20-HETE stimulation.** It has previously been suggested that the increase in tension induced by 20-HETE in guinea pig ASM may involve Ca\(^{2+}\) mobilization from an intracellular pool, according to the positive inotropic transient response recorded in the absence of extracellular Ca\(^{2+}\) (8). Hence, the effects of various intracellular Ca\(^{2+}\) channel blockers and modulators were tested to verify their possible effect on responses to 20-HETE. As can be seen in Fig. 1, the time course of response to 20-HETE on guinea pig ASM following preincubation with ryanodine (Fig. 1B) was similar to that obtained with 1 μM 20-HETE alone (Fig. 1A), confirming the notion that mobilization of the ryanodine-sensitive pool, as assessed by the slow increase in resting tone, does not modify the isotropic responses to 20-HETE.

Tension induced by 20-HETE was characterized by a rapid rise, followed by a sustained plateau phase that slowly inactivated over time (Fig. 1C). The effects of 20-HETE were fully reversible upon washout with fresh Krebs solution (Fig. 1, A and C). In contrast, preincubation with 36 μM 2-APB, an InsP\(_3\) receptor inhibitor (19), prevented any subsequent response to 20-HETE (Fig. 1D and Table 1, n = 8). When added alone, 2-APB induced a biphasic response, starting with a low-amplitude increase in tension followed by a long-lasting relaxation.

Figure 1E shows that preincubation with 1 μM thapsigargin, a blocker of sarco/endoplasmic reticulum calcium ATPase (SERCA), resulted in a leakage of calcium from intracellular Ca\(^{2+}\) stores, as attested by tone increase, and a reduction in the response to 1 μM 20-HETE. However, the subsequent trace recorded on the same tissue (Fig. 1F) confirms that thapsigargin preincubation did not impair the response to 80 mM K\(^+\) in modified Krebs solution. On the other hand, the response to the high-[K\(^+\)] solution was blocked by 1 μM nifedipine (a L-type Ca\(^{2+}\) channel blocker, Ref. 20). Quantitative data analysis and corresponding n values are presented in Table 1. Altogether, these data suggest that the positive isotropic effects induced by 20-HETE on guinea pig ASM require the integrity of the InsP\(_3\)-sensitive intracellular Ca\(^{2+}\) release pathway.

Complementary experiments attest that under control conditions, two sequential 20-HETE challenges basically produced the same isotropic responses, as illustrated and quantified in Fig. 2. A and B, respectively. However, a decrease in basal tone might modify the tonic response to the same concentration of 20-HETE. Figure 2C shows that a 500-mg reduc-
tion of the tone reduced the amplitude of the responses to 20-HETE by 61%.

To test the effects of the sequential additions of 2-APB and ryanodine, additional experiments were performed. Independently of the order of addition of 36 μM 2-APB and 10 μM ryanodine, the effect of 1 μM 20-HETE on the ASM tone was abolished (Fig. 2, D and E). The effect of either inhibitor was consistent with what we observed in the previous experiments (Fig. 1, B and D).

Activation of selective and nonselective Ca2+ channels supports the plateau of 20-HETE responses. In a previous report, the inotropic effects induced by 20-HETE on guinea pig ASM were fully inhibited by cumulative addition of 100 μM GdCl3 (a nonselective Ca2+ channel blocker) and 1 μM nifedipine (8). In the present study, we verified that the addition of these two blockers in reverse order also relaxes 20-HETE responses. Figure 2A shows that 1 μM nifedipine relaxed the inotropic response induced by 1 μM 20-HETE. This nifedipine-induced relaxation reached 75% of the mechanical response (Table 2),

Table 1. Changes in intracellular Ca2+ pool status modify the responses to 20-HETE

<table>
<thead>
<tr>
<th>Compound</th>
<th>Tension Developed, mg</th>
<th>%</th>
<th>n</th>
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<tbody>
<tr>
<td>1 μM 20-HETE</td>
<td>187.2 ± 26.7</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>10 μM Ryanodine</td>
<td>592.1 ± 65.6</td>
<td>(316.3)*</td>
<td>12</td>
</tr>
<tr>
<td>Ryanodine + 20-HETE</td>
<td>221.3 ± 67.9</td>
<td>118.2†</td>
<td>12</td>
</tr>
<tr>
<td>36 μM 2-APB</td>
<td>-203.8 ± 53.8‡</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2-APB + 20-HETE</td>
<td>0.0</td>
<td>0.0</td>
<td>8</td>
</tr>
<tr>
<td>1 μM Thapsigargin</td>
<td>392.0 ± 64.8</td>
<td>(209.4)*</td>
<td>5</td>
</tr>
<tr>
<td>Thapsigargin + 20-HETE</td>
<td>35.6 ± 11.2</td>
<td>19.3†</td>
<td>5</td>
</tr>
</tbody>
</table>

Comparative effects of 1 μM 20-hydroxyeicosatetraenoic acid (20-HETE) alone and following pretreatment with the various compounds ryanodine (10 μM), 2-aminoethoxydiphenyl borate (2-APB, 36 μM) and thapsigargin (1 μM). See Fig. 1, A–C, for corresponding illustrations. *Percentage of mechanical tension induced by inhibitors in the absence of 20-HETE. †Percentage of tension induced by 20-HETE. ‡Note that 2-APB consistently induced a biphasic effect, resulting in a relaxation, as attested by the negative value.
whereas subsequent addition of 100 μM GdCl₃ relaxed the remaining muscle tension. Table 2 summarizes the independent and cumulative effects of these two compounds. Thus the active tension induced by 20-HETE was fully reversed by the combined addition of selective and nonselective cationic channel blockers. However, neither nifedipine nor Gd³⁺ relaxed basal tone (data not illustrated).

Effect of a vanilloid receptor antagonist on the inotropic effect of 20-HETE. It has recently been proposed that anandamide and a number of lipoxygenase products of the AA cascade activate VR1, the first identified member of the TRPV subfamily (3). Our group has also shown that the tonic responses induced by 20-HETE mainly involved the activation of TRPC channels (8) and more specifically the activation of TRPC6 (2). We thus postulated that, in a native tissue such as guinea pig ASM, the sustained response phase to 20-HETE may be inhibited by putative inhibitors of the TRP channel superfamily. In the absence of specific TRP channel blockers, however, neither nifedipine nor Gd³⁺ relaxed basal tone (data not illustrated).

**Table 2. Inhibitory effects of sequential addition of nifedipine and Gd³⁺ on 20-HETE-induced tension in guinea pig airway smooth muscle**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tension Developed, mg</th>
<th>Inhibition, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 μM 20-HETE</td>
<td>171.3 ± 32.5</td>
<td>0.0</td>
<td>7</td>
</tr>
<tr>
<td>Addition of 1 μM nifedipine</td>
<td>75.7 ± 9.6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Addition of 100 μM Gd³⁺</td>
<td>28.3 ± 5.9</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Addition of nifedipine + Gd³⁺</td>
<td>100.4 ± 7.7</td>
<td>7</td>
<td></td>
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Values are means ± SE. Cumulative addition of nifedipine and Gd³⁺ completely abolished the 20-HETE inotropic responses.
20-HETE on guinea pig ASM, but it also relaxed the cumulative contractions induced by both 1 μM 20-HETE and 0.05 μM capsaicin, a VR1 agonist (Fig. 3C). The concentration-dependent effect of capsazepine on 1 μM 20-HETE-induced mechanical tension is demonstrated in Fig. 4A. Quantitative results reveal that 0.3 μM capsazepine relaxed the mean response to 20-HETE by 47.6 ± 11.4% (n = 8, Fig. 4B).

Capsazepine pretreatment modifies the time course of 20-HETE responses. Preincubations with capsazepine were used to assess whether this vanilloid receptor antagonist mainly affects the tonic responses to 20-HETE. Figure 5 shows three sequential traces recorded on the same tissue. An initial control trace was first obtained on 1 μM 20-HETE pharmacological stimulation for assessment of ASM reactivity as well as the time course of the inotropic response (Fig. 5A). After eicosanoid washout, the tissue was incubated with 10 μM capsazepine, inducing a biphasic response: an initial yet unexplained transient increase in tension followed by a slow and prolonged relaxation phase (Fig. 5B). Subsequent 20-HETE challenges consistently triggered transient responses. All trials yielded similar responses, namely suppression of the plateau phase by capsazepine pretreatment (Fig. 5B). Figure 5C shows that this effect was fully reversible following a 20-min washout period in Krebs solution and a final 20-HETE challenge. Quantitative analysis revealed that the presence of capsazepine did not significantly alter the rate of rise or amplitude of 20-HETE inotropic responses. On the other hand, Fig. 5D clearly demonstrates that capsazepine pretreatments inhibited the tonic component in a concentration-dependent manner (1, 3, and 10 μM). These results indicate that the plateau of 20-HETE responses is most likely supported by the activation of ionic conductances sensitive to the vanilloid antagonist.

Concurrent experiments were performed in normal and Ca²⁺-free Krebs solution (Fig. 6, A–C). The bronchia were initially challenged with 1 μM 20-HETE in control conditions (Fig. 6A), whereas Fig. 6B illustrates the typical relaxing effect.
The first finding in the present study is the demonstration that increases in mechanical tension induced by 20-HETE involve a process of intracellular Ca\(^{2+}\)-release occurring through InsP\(_3\) receptors as well as from a thapsigargin-sensitive pool, a process likely triggered by an unidentified GPCR. However, the addition of 2.5 mM CaCl\(_2\) triggered a large increase in mechanical tension, possibly related to an increase in extracellular Ca\(^{2+}\) concentration and Ca\(^{2+}\) entry through Ca\(^{2+}\) conducting channels. This positive inotropic response was followed by a rapid relaxation due to the presence of 1 μM capsazepine in the medium. After washout with normal Krebs solution and final stimulation with 1 μM 20-HETE, tissues consistently displayed biphasic inotropic responses (Fig. 6C). Such unexpected biphasic behavior underscores a partial uncoupling (dephasing) between rapid Ca\(^{2+}\) release and delayed Ca\(^{2+}\) entry during the recovery process. The latter may involve additional and specific biochemical steps.

Effects of DAG-lipase and CYP-450 \(\omega\)-hydroxylase inhibitors. To probe the putative intracellular processes involved in the pharmacomechanical coupling following agonist stimulation, two independent sets of experiments were performed to evaluate the role of DAG-lipase and CYP-450 \(\omega\)-hydroxylase following repetitive CCh and 20-HETE challenges. Taking into account that sequential activations of these enzymes can lead to intracellular production of AA and 20-HETE (see Fig. 9), we used RHC-80267 and ETYA as selective DAG-lipase and CYP-450 \(\omega\)-hydroxylase inhibitors, respectively. Figure 7, A–E, demonstrates that 35 μM RHC-80267 decreased the tonic response to CCh. Moreover, subsequent addition of RHC-80267 in association with 10 μM ETYA largely impaired CCh responses. Nevertheless the inhibitory effects were reversed upon washout of inhibitors and final muscarinic challenge. These results suggest that AA and 20-HETE synthesis may occur following muscarinic receptor activation.

Similar experiments were performed to assess the effect of both inhibitors RHC-80267 and ETYA on the 20-HETE responses (Fig. 8, A–F). RHC-80267 (35 μM) exhibited a relaxing effect on the basal tone (Fig. 8B). Also, it has a facilitating effect on the responses triggered by exogenous 20-HETE (Fig. 8B) compared with the control response (Fig. 8A). However, the addition of 35 μM RHC-80267 and 10 μM ETYA, a poorly metabolized AA analog, consistently prevented the development of any inotropic responses normally triggered by exogenous addition of 1 μM 20-HETE (Fig. 8C). We also demonstrate that the effects of the enzymatic inhibitors were clearly reversible (Fig. 8D). To prove that the combined effects of the inhibitors were mainly due to ETYA, the preparations were pretreated with 10 μM ETYA before 20-HETE challenge. This addition of ETYA induced a large inhibitory effect on the eicosanoid responses (Fig. 8E). The quantitative results (Fig. 8F) confirm that the inhibitory effects were mainly due to ETYA pretreatments.

To test the putative involvement of PLA\(_2\), the alternative pathway for intracellular AA production upon 20-HETE stimulation, complementary experiments were performed in the presence of ONO-RS-082, a PLA\(_2\) inhibitor, either in association or not with ETYA. Our data demonstrate that ONO-RS-082 induced a 64.4% \((n = 4)\) inhibition of 20-HETE responses, whereas the association of ONO-RS-082 and ETYA inhibited 86.2% \((n = 4)\) of the inotropic responses to 20-HETE. Upon washout of the inhibitors, only a partial recovery of 20-HETE responses was recorded, after 30 min in fresh Krebs solution. In contrast, the CCh response at the end of the experiment, following washout of the PLA\(_2\) inhibitors, was similar 109 ± 12% to the control value (100%). Thus an alternative pathway is proposed to explain the production of intracellular arachidonic acid via PLA\(_2\) activation, likely by a putative eicosanoid receptor for 20-HETE and a concomitant increase in free Ca\(^{2+}\) (Fig. 9).

DISCUSSION

The first finding in the present study is the demonstration that increases in mechanical tension induced by 20-HETE involve a process of intracellular Ca\(^{2+}\)-release occurring through InsP\(_3\) receptors as well as from a thapsigargin-sensitive pool, a process likely triggered by an unidentified GPCR. In addition, the sustained pharmacomechanical response, which was previously shown to be related to activation of TRPC channels in various smooth muscle cells (8, 16, 23), was either largely abolished or reversed by micromolar concentrations of capsazepine, an inhibitor of the type 1 vanilloid receptor, VR1 or TRPV1 (22). However, the cumulative effects of 20-HETE and capsaicin, a vanilloid agonist, were...
additive, suggesting that they may occur via different sets of receptors and effectors. This is the first evidence that the sustained pharmacological response to this eicosanoid is highly sensitive to capsazepine inhibition. This observation may provide a pharmacological tool of putative experimental interest to prevent the physiological effects of 20-HETE.

The second major finding of this study is that intracellular AA synthesis, as well as its metabolite, may be involved in the control and activation of transmembrane effectors, such as summarized in Fig. 9.

**Mode of action of 20-HETE.** It has been demonstrated that inotropic responses to various concentrations of 20-HETE in ASM cells involve the activation of an intracellular Ca^{2+} release process, followed by sustained Ca^{2+} entry, which rapidly reverses upon 20-HETE washout (8). Previous experiments, performed in Ca^{2+}-free solution, have shown that 20-HETE triggers transient increases in tension, which significantly differs from typical responses recorded in the presence of normal extracellular Ca^{2+} concentrations (2.5 mM) reported above in control conditions. These results led to a complementary set of experiments to determine which type of Ca^{2+} release pathway was activated by this exogenously added eicosanoid. The use of pharmacological inhibitors of InsP_{3} receptors and SERCA pumps, namely 2-APB and thapsigargin, respectively, attests to the key role of these specific intracellular Ca^{2+} release and uptake systems in the overall pharmaco-mechanical response triggered by 20-HETE. Hence, whereas ryanodine alone had an effect on resting tone, it had no effect on 20-HETE responses (Fig. 1B). It was also found that this tonic response was never observed in the presence of 2-APB, suggesting that an intact InsP_{3}-mediated process is required to yield the sustained plateau phase. However, the selectivity of 2-APB for InsP_{3} has been questioned (18). This compound may inhibit Ca^{2+} entry through nonselective cationic channels.

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**Fig. 5.** Capsazepine pretreatment modulates the contractile response to 20-HETE. A–C: sequential recordings obtained on the same tissue in normal Krebs solution. A: positive inotropic response induced by 1 μM 20-HETE in control conditions. B: 10 μM capsazepine pretreatment induced a slow biphasic effect on resting tone and transformed the 1 μM 20-HETE response into a transient contraction. C: recovery upon washout of the tissue, followed by 1 μM 20-HETE challenge. There was full recovery of the sustained plateau. D: quantification of the concentration-dependent effect of capsazepine pretreatment on the sustained portion of the 20-HETE induced tone. The reported values represent series of single data points, 5 min after the peak response to 1 μM 20-HETE, either in the absence (open bars) or in the presence of capsazepine (filled bars). Data are expressed as a percentage of inhibition of the peak responses. Increasing concentrations of capsazepine did not significantly alter the time to reach the peak responses. *P < 0.05; n values are in parentheses.
of the TRPC subfamily, as previously proposed (18). Complementary experiments, involving sequential additions of the Ca\(^{2+}\)/H\(_{11001}\) release channels inhibitors, also prevented 20-HETE induced responses (Fig. 2, D and E).

Recent studies also suggest that 20-HETE acts through the activation of surface membrane conductances, related to the TRPC channel subfamily, as demonstrated at the cellular, membrane, and molecular levels (2, 8). Moreover, microelectrode measurements have revealed that submicromolar concentrations of 20-HETE induce small, but significant, depolarization of the resting tone (1 g). In low [Ca\(^{2+}\)]\(_{o}\), 1 μM capsazepine had no effect on tone; however, 1 μM 20-HETE induced a transient low-amplitude response. Upon addition of 2.5 mM CaCl\(_2\), a large, although transient, contraction was consistently observed. C: recovery of 20-HETE response upon tissue superfusion with normal Krebs solution (2.5 mM CaCl\(_2\)) and eicosanoid challenge. Note the biphasic inotropic response and faster relaxation, compared with the time course of the contractile response in A.

**The capsazepine connection.** The data presented herein consistently and conclusively show that capsazepine, a VR1 antagonist, reverses the tonic inotropic effects of 20-HETE in a concentration-dependent manner but does not affect transient responses induced by this eicosanoid. These transient responses most likely rely on the activation of undisclosed GPCR and a Ca\(^{2+}\) release process mediated by InsP\(_3\) receptors, as discussed above and summarized in Fig. 9. Initially, the experiments were designed to demonstrate that the effects of 20-HETE were not mediated by the activation of TRPV channels, whose selectivity to divalent cations has been reported to be slightly higher than that reported for nonselective TRPC channels (7, 22). Our unexpected, although consistent, results led us to further our investigations through the use of capsazepine. The fact that capsazepine completely reversed the cu-
The cumulative effects of 20-HETE and capsaicin, regardless of the additive order (data not illustrated), strongly suggests that the vanilloid antagonist has a broader spectrum of inhibition than initially thought. The present results raise the possibility that, in ASM cells, capsazepine may interact and basically inhibit various types of nonselective membrane conductances. This possibility was also raised in another study by Watanabe et al. (29) on TRPV4, although the authors subsequently demonstrated that intracellular production of EET, rather than 20-HETE, upregulated TRPV4 isoform in an overexpressing system and cultured endothelial cells (29). In contrast, it was recently reported that in cerebellar Purkinje cells, the activation of TRPC1 cation channels occurs through stimulation of metabotropic glutamate receptors (15). These reports as well as observations from the present study suggest that members of the TRP superfamily could represent either inotropic effectors of previously characterized metabotropic GPCRs (7) or inotropic receptors for the so-called orphan agonists, already characterized for their physiological effects (13, 32). Consequently, eicosanoids such as 20-HETE and EET (4), which act as autocrine, paracrine, and likely intracrine modulators (25), would certainly fulfill the latter definition.

An alternative explanation for the reported inhibitory effects of the vanilloid antagonist would be that capsazepine may block L-type Ca<sup>2+</sup> channels and associated Ca<sup>2+</sup> influx as proposed in rat ileum (20). However, according to our quantitative analysis, 1 μM capsazepine relaxes 100% of the responses to 20-HETE, whereas 1 μM nifedipine blocks 75% of tonic responses induced by 1 μM 20-HETE and Gd<sup>3+</sup> the remaining 25% (Table 2). Thus it appears unlikely that the potent inhibitory effects of capsazepine specifically occur through inhibition of L-type Ca<sup>2+</sup> channels. A third alternative explanation would be that exogenous addition of 20-HETE promotes the synthesis and/or release of a vanilloid agonist in the challenged tissues. Its effect would hence be antagonized by the pharmacological addition of capsazepine, although to date, there are no data to either support or rule out this hypothesis.

**Alternative signaling pathways.** If 20-HETE does indeed activate TRP channels by a GPCR, as previously proposed (25) and evoked above, there is a second and more evocative possibility that could be described as post-GPCR signaling processes, namely a complementary cascade of putative mechanistic events. Fig. 9. Functional scheme summarizing the mode of action of 20-HETE on transmembrane and intracellular mechanisms in ASM cells. G, trimeric G protein; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PLC, phospholipase C; AA, arachidonic acid; CYP-450 ω-H, cytochrome P-450 ω-hydroxylase. InsP<sub>3</sub>, inositol (1,4,5)-triphosphate; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; RE, endoplasmic reticulum; RS, sarcoplasmic reticulum; IK<sub>Ca</sub>, calcium-activated potassium channels; TRPV1, type 1 vanilloid receptor. Note that exogenous agonists and 20-HETE can activate InsP<sub>3</sub> receptors, as well as AA production (either by DAG-lipase or PLA<sub>2</sub> pathways) and 20-HETE synthesis, leading to the activation of transient receptor potential (TRP) channels. According to our experimental results (Figs. 7 and 8) following CCh stimulus, AA production and intracellular eicosanoid synthesis would result from DAG-lipase and CYP-450 ω-hydroxylase activation, while exogenous 20-HETE addition would result in PLA<sub>2</sub> activation and alternative AA production. Consequently, endogenous 20-HETE would also be produced within the intracellular compartment and processes. ΔV<sup>+</sup>, depolarization; ΔV<sup>−</sup>, hyperpolarization.
anistic interest as described in the functional scheme illustrated in Fig. 9.

To date, only DAG and its synthetic analog, 1-oleyl-2-acetyl-sn-glycerol, are recognized as intracellular TRP channel activators (11). We propose that 20-HETE may also act as an intracellular ligand for nonselective cationic channels. To support this view, we have shown that CCh-induced responses are largely reduced by the combined addition DAG-lipase and CYP-450 inhibitors (Fig. 7), whereas exogenous responses to 20-HETE are not inhibited by RHC-80267 but are completely abolished by ETYA (Fig. 8). These observations challenge, but do not rule out, the direct activation of TRP channels by DAG (6, 7). Because RHC-80267 does not reduce eicosanoid responses, it could be postulated that increased production of AA by exogenous 20-HETE may result from PLA2 activation, as strongly supported by the inhibitory effects quantified in the presence of ONO-RS-082 and ETYA. These results provide new insights toward a better understanding of the gating mechanism of several members of the TRP superfamily. This activation would involve either interfacial or allosteric interactions, related to subtle modifications of the lipidic environment in proximity of the channel proteins, as recently suggested in a didactical review (7).

Putative role of Rho-kinase. It has recently been reported that contraction induced by 20-HETE on small coronary arteries depends on the activation of the Rho-kinase pathway (24). The activation of signal transduction through G protein Rho-kinase and protein phosphatase has been proposed for several agonists in various smooth muscle tissues (27). To date we have not tested nor obtained any data supporting or negating this issue in ASM cells. However, we have previously shown that the pharmacological responses triggered by 20-HETE in ASM were not modified by GF-109203X, a PKC inhibitor (8), which basically rules out the involvement of this biochemical pathway in the present scheme (Fig. 9).

Physiological relevance in ASM. With regard to the functional relevance of the present observations in ASM cells, there are two putative biochemical implications to the present results: TRPC and TRPV subunits may form heteromultimer complexes that are sensitive to (or regulated by) the local production of specific eicosanoids in either normal or pathological conditions. This hypothesis is supported by previous reports attesting to the existence of TRPC1/TRPC3 heteromultimers as well as TRPC1/TRPC4 and TRPC1/TRPC5 heteromultimers (6, 28). On the other hand, it has been proposed that TRP members of the vanilloid subfamily, whose distribution often overlap, may also heteromultimerize with each other, more specifically TRPV1 and TRPV3 in neuronal tissues (7). However, this possibility remains to be assessed in ASM tissues.

In conclusion, the data presented herein describe a novel and unexpected inhibitory mechanism by which a vanilloid antagonist, capsaicin, likely interacts with surface membrane conductances, which in turn control Ca2+ entry in ASM cells during the plateau phase of the tonic responses induced by the eicosanoid HETE-20, which has received much attention due to its vascular and pulmonary effects (31). Because it has been shown that leukotriene D4 also activates nonselective cationic currents (26), it is anticipated that capsaicin may also abolish the tonic responses induced by various agents, including leukotrienes, although this hypothesis remains to be tested. On the other hand, in vivo, 20-HETE is likely generated by the cascade summarized herein (Fig. 9) via a mechanism previously unspecified until now. These results clearly shed new light on the complex mode of action of eicosanoids, such as 20-HETE, and raise new questions regarding their putative cross-reactivity with other members of the TRP family (7).

This work has never been communicated elsewhere and represents an original set of data.

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