Relaxing Effects of NO Donors on Guinea Pig Trachea In Vitro are Mediated by Calcium-Sensitive Potassium Channels

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ABSTRACT
The relaxing effects of the nitric oxide (NO) donors 1,2,3,4-oxatriazolium,3-(3-chloro-2-methylphenyl)-5-[(4-methoxyphenyl)sulfonyl]amino]-hydroxide inner salt (GEA 3268) 1,2,3,4-oxatriazolium,3-(3-chloro-2-methylphenyl)-5-[methylsulfonyl]amino]-hydroxide inner salt (GEA 5145), 3-morpholinosydnonimine (SIN-1) and S-nitroso-N-acetylpenicillamine (SNAP) were inhibited in vitro by iberiotoxin (IbTX) and charybdotoxin (ChTX), the two selective inhibitors of Ca++-activated K+ channels (KCa) in guinea pig trachea. When studied in cumulative concentrations in metacholine contraction, the relaxing effects of the NO donors were inhibited by at least 70% in the presence of the toxins, with the exception of SIN-1 in the presence of ChTX. The inhibitory effect of ChTX was less marked than that of IbTX. This suggests that the relaxing effects of the structurally different NO donors are mediated through KCa channels and that IbTX is more potent than ChTX. A selective inhibitor of soluble guanylate cyclase, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ), significantly inhibited the relaxing effects of GEA 3268 and GEA 5145 on metacholine and KCl constriction and almost totally inhibited the relaxing effects of SIN-1 and SNAP. The inhibitor of the delayed rectifier K+ channel current 4-aminopyridine did not influence the relaxations of the NO donors, and under the experimental conditions of this study, the ATP-sensitive K+ channel inhibitor glibenclamide had no effect. In conclusion, the relaxing effects of the structurally different NO-releasing compounds are mediated via KCa channels. However, the significance of some other possible mechanisms unrelated to K+ channels cannot be excluded.

We have previously shown that the relaxing effects of the two new experimental NO donors GEA 3268 and GEA 5145 (oxatriazole sulfonylamides) (fig. 1) are more potent than those of sodium nitroprusside and SIN-1 in the bronchi of guinea pigs and rats in vitro (Vaali et al., 1996). These compounds can produce nitrates (NO2) and nitrates (NO3), thus mediating the relaxing effects through the cGMP-mediated pathway. Other possible mechanisms for the relaxing effects, such as the opening of KCa channels, were investigated in the present study. KCa channels exist in high density in smooth muscle plasmalemma and have a large unitary conductance; they may therefore contribute to the maintenance of the resting membrane potential in some smooth muscle preparations (Edwards and Weston, 1994). KCa are both Ca++- and voltage-dependent. Their large single-channel conductance and localization on the neurons and on secretory and muscle cells suggest that they are a key element in the control of cellular excitability (Petersen and Maruyama 1984; Rudy, 1988; Brayden and Nelson, 1992; Stretton et al., 1992). In particular, agents that increase KCa activity, during or after the events that increase intracellular Ca++, would be expected to reduce the cellular excitability and could directly or indirectly reduce neurotransmitter and hormone release (Gribkoff et al., 1996). Adrenergic β-receptor stimulation results in an increase in KCa channel activity in airway smooth muscle cells (Kume et al., 1989). These channels play an important functional role in bronchodilation (Jones et al., 1990; Miura et al., 1992) and are thus interesting from the standpoint of asthma drug development. KCa channel openers relax the airway smooth muscle of several species, including the guinea pig (Allen et al., 1986; Arch et al., 1988), bovine (Gater, 1989; Longmore et al., 1991)

ABBREVIATIONS: ANOVA/MANOVA, analysis of variance; 4-AP, 4-aminopyridine; [ATP], cellular ATP concentration; KATP channels, ATP-sensitive K+ channels; ChTX, charybdotoxin; DMSO, dimethylsulfoxide; GEA 3268, 1,2,3,4-oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[(4-methoxyphenyl)sulfonyl]amino]-hydroxide inner salt; GEA 5145, 1,2,3,4-oxatriazolium, 3-(3-chloro-2-methylphenyl)-5-[methylsulfonyl]amino]-hydroxide inner salt; IbTX, ibetoxitin, KCa channels, Ca++-activated K+ channels; Kd, delayed rectifier channels; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one; sGC, soluble guanylate cyclase; SIN-1, 3-morpholinosydnonimine; SNAP, S-nitroso-N-acetylpenicillamine.

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10
Materials and Methods

Isolated trachea of the guinea pigs. English short-haired tricolored guinea pigs (350–450 g) of both sexes, bred at the Public Institute of Health, Helsinki, were decapitated and their trachea cut into pieces and mounted in an 8-ml organ chamber containing Krebs-Ringer solution of the following composition (mM): NaCl 119, NaHCO3 25, glucose 11.1, CaCl2 × H2O 1.6, KCl 4.7, KH2PO4 1.2, MgSO4 × 7 H2O 1.2. The pH was adjusted to 7.4. The solution was aerated with 96% O2 and 4% CO2 during the experiments, and the resting tension was set at 1.5 g. All the experiments were carried out in the presence of indomethacin (3.3 μM), propranolol (1 μM), Nω-nitro-arginine (100 μM) and phentolamine (10 μM). Indomethacin was present throughout the experiment in order to prevent the fading of neural response as a result of endogenous prostaglandin production. Phentolamine, propranolol and Nω-nitro-arginine were added to inhibit alpha and beta adrenergic responses and in order to observe the effects of exogenous NO responses. The effects of ODQ, as well as those of the Kᵦ channel inhibitors, were studied after the submaximal constriction (1 μM metacholine or 40 mM KCl) of the tracheal ring had reached its plateau and were calculated as a percentage of the maximal relaxation. The pretreatment time of the inhibitor was 20 min, after which the NO-donating drug or vehicle was added cumulatively at 6-min intervals. The changes in tension were recorded with Grass force-displacement transducers and amplifiers (FT03, Grass Medical Instruments, Quincy, MA).

Drugs. Drugs from the following sources were used: GEA 3268, GEA 5145 (fig. 1) and SIN-1 were synthesized by A/S GEA Farmaceutisk Fabrik (Hvidovre, Denmark). Glibenclamide was a kind gift from Orion Ltd, (Espoo, Finland). Nω-nitro-arginine and ODQ came from Alexis (Läufelfingen, Switzerland). Metacholine (acetyl-β-methylcholine chloride), indomethacin, propranolol hydrochloride, phentolamine hydrochloride, Britx, Chtx, 4-AP and SNAP came from Sigma Chemicals, (St. Louis, MO); reagents for the Krebs-Ringer came from Riedel-de Haén, (Seelze, Germany). The Krebs-Ringer solution was prepared in ultrapure water (Milliq, Millipore, Bedford, MA). Indomethacin and phentolamine were dissolved in absolute ethanol, the final concentration of ethanol in the baths being 0.004%; Nω-nitro-L-arginine was dissolved in 0.1 M HCl; GEA 3268, GEA 5145, glibenclamide and ODQ were dissolved in DMSO, the final concentration of DMSO in the baths being not more than 0.05%. All the other reagents were dissolved in water. The total number of guinea pigs used in the study was 60.

Statistical analysis. The data are presented as means ± S.E.M.; n = 4 to 12. Analysis of variance (ANOVA/MANOVA) was used with the program Statistica, release 4.5, 1993 (Statsoft, Inc.; Tulsa, OK) followed by the Newman-Keuls’ test for multiple comparisons. The statistical significance of differences were *P < .05, **P < .01 and ***P < .001.

Results

Inhibition of the sGC. The maximal relaxations induced by GEA 3268 (10 μM), GEA 5145 (10 μM), SIN-1 (33 μM) and SNAP (100 μM) in the absence of ODQ were approximately 90%, 90%, 70% and 70% after 1 μM metacholine. In the presence of ODQ (1 μM), the relaxing effects of GEA 3268 (10 μM), GEA 5145 (10 μM), SIN-1 (33 μM) and SNAP (100 μM) were 60%, 40%, 20% and 13%, and in the presence of ODQ (33 μM), they were approximately 9%, 30%, 0% and 0%, respectively (fig. 2). In 40 mM KCl, ODQ also reduced the relaxation percentage dose-dependently (table 1). The strong inhibitory effect of ODQ suggests that NO mediates the relaxing effects of the compounds through sGC.

The effects of 4-AP and glibenclamide on relaxation. All the effects of 4-AP (0.1 and 1 mM) on the relaxations of the NO donors studied were minor, so only the EC50 values are presented in table 2.

Fig. 2. Relaxing effects of the NO donors in cumulative concentrations on the metacholine (1 μM)-constricted guinea pig trachea in vitro. The samples were incubated with a combination of indomethacin (3.3 μM), propranolol (1 μM), NO2-arginine (100 μM) and phentolamine (10 μM). Statistically significant differences in the responses are calculated by ANOVA/MANOVA followed by the Newman-Keuls’ test for the multiple comparisons. The results are given as the mean ± S.E.M. and compared with the controls (*P < .01 and **P < .001). N = 7 to 12 for the controls (○) and the inhibitory effect of ODQ 1 μM (●), and 33 μM (□) for GEA 3268, GEA 5145, SIN-1 and SNAP. N = 7 to 9 for GEA 5145 in 1 μM ODQ. N = 4 for the remainder.
KATP channels are closed. After KCl constriction, glibenclamide (33 nM) significantly increased the relaxing effects of GEA 3268 when the KATP channels are closed. After KCl constriction, the efficacy of GEA 3268 increased by 20% in the presence of glibenclamide (table 1), which suggested that the relaxing effects of GEA 3268 increase when the KATP channels are closed. After KCl constriction, glibenclamide had no effect on the relaxation of the NO donors (fig. 3, table 2).

**Inhibition of relaxation by IbTX and ChTX.** IbTX (33 nM and 100 nM) prevented the relaxing effects of all the NO donors concentration-dependently after metacholine constriction (fig. 4). Relaxations induced by GEA 3268, GEA 5145, SIN-1 and SNAP were inhibited 70%, 80%, 60% and 60%, respectively, by 100 nM IbTX. ChTX had no effect on SIN-1 relaxation: The final SIN-1 relaxation was 72% ± 4%; and in the presence of 33 nM and 100 nM ChTX, the relaxations were 62% ± 4% and 70% ± 8%, respectively, whereas the effects of all the other NO donors were significantly inhibited. In the presence of 33 and 100 nM ChTX, the relaxations of GEA 3268, GEA 5145 and SNAP were inhibited approximately by 20%, 40% and 20% and by 50%, 55% and 35%, respectively (fig. 5). Both the toxins were able to inhibit dose-dependently the relaxations of the NO donors studied, except in the presence of ChTX and SIN-1; thus it is obvious that the relaxing effects of these compounds are mediated at least partly by the Kca channels. However, the inhibitory effect of ChTX was less than that of IbTX (figs. 4 and 5).

When KCl was used as the constricting agent, IbTX (33 nM and 100 nM) inhibited relaxations induced by GEA 3268 (10 μM), SIN-1 (33 μM) and SNAP (100 μM) significantly and concentration-dependently (table 1). ChTX (33 nM) concentration had no effect on the relaxations but in 100 nM concentration had significant effects on the SIN-1- and SNAP-induced relaxations (table 1). The moderate relaxing effects of all the NO donors in the presence of 100 nM IbTX or ChTX suggest that part of the relaxing mechanism is independent of the Kca channels.

**Discussion**

In the present study, GEA 3268, GEA 5145, SIN-1 and SNAP relaxed the metacholine- and KCl-precontracted guinea pig trachea, which is consistent with the relaxing effects of those NO donors used in the guinea pig and rat airway smooth muscle (Vaali et al., 1996). ODQ, a selective inhibitor sGC, inhibited concentration-dependently the relaxations induced by all the NO donors in the methacholine- and KCl-precontracted trachea preparations. ODQ (1 μM) inhibited the relaxation induced by SIN-1 and GEA 5145 more than the relaxation induced by GEA 3268, which is consistent with the fact that SIN-1 and GEA 5145 are stronger releasers of nitrates and nitrates than GEA 3268 (Vaali et al., 1996). ODQ has been shown to inhibit NO-induced increases in cGMP concentrations in the brain (Garthwaite et al., 1995). ODQ also selectively inhibits sGC in the vascular tissue and platelets without having any effect on NO (Mor et al., 1996), and it inhibits the relaxations induced by electrical field stimulation and sodium nitroprusside in the rabbit anococcygeus muscle, as well as inhibiting the basal electrical field stimulation and NO-stimulated production of cGMP (Cellek et al., 1996). Thus this compound, which neither inactivates NO nor inhibits its release, acts as a selective inhibitor of sGC in the brain, platelets and smooth muscle. Taken together, our results indicate that the NO donors relax the guinea pig trachea through a cGMP-dependent pathway.

The Kca currents are relatively insensitive to TEA (1 mM in single-channel experiments) and are unaffected by ChTX.

### Table 1

<table>
<thead>
<tr>
<th>sGC Inhibitor or K⁺ Channel Inhibitors</th>
<th>GEA 3268 (10 μM) Maximum Relaxation (%)</th>
<th>GEA 5145 (10 μM) Maximum Relaxation (%)</th>
<th>SIN-1 (33 μM) Maximum Relaxation (%)</th>
<th>SNAP (100 μM) Maximum Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>55.6 ± 5.2</td>
<td>67.9 ± 4.9</td>
<td>44.6 ± 4.1</td>
<td>40.5 ± 4.0</td>
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<tr>
<td>ODQ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 μM</td>
<td>32.2 ± 6.4**</td>
<td>22.9 ± 2.6***</td>
<td>6.7 ± 1.7***</td>
<td>10.9 ± 2.7***</td>
</tr>
<tr>
<td>33 μM</td>
<td>15.9 ± 2.4***</td>
<td>19.6 ± 2.9***</td>
<td>5.5 ± 2.5***</td>
<td>6.0 ± 2.1***</td>
</tr>
<tr>
<td>Glibenclamide 33 μM</td>
<td>74.4 ± 4.5**</td>
<td>77.8 ± 7.4</td>
<td>47.3 ± 4.6</td>
<td>43.9 ± 2.4</td>
</tr>
<tr>
<td>IbTX</td>
<td></td>
<td></td>
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<tr>
<td>33 nM</td>
<td>42.5 ± 5.9</td>
<td>45.6 ± 6.2*</td>
<td>25.6 ± 3.0**</td>
<td>24.1 ± 4.6**</td>
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<tr>
<td>100 nM</td>
<td>35.5 ± 12.6*</td>
<td>56.1 ± 2.1</td>
<td>19.2 ± 2.2*</td>
<td>22.3 ± 1.8**</td>
</tr>
<tr>
<td>ChTX</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>33 nM</td>
<td>60.9 ± 3.5</td>
<td>57.8 ± 4.3</td>
<td>55.4 ± 4.3</td>
<td>37.0 ± 7.0</td>
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<tr>
<td>100 nM</td>
<td>49.7 ± 3.8</td>
<td>55.5 ± 3.7</td>
<td>30.3 ± 1.7*</td>
<td>27.8 ± 2.4*</td>
</tr>
</tbody>
</table>

Fig. 3. The effect of glibenclamide 33 μM (●) on the NO donors in metacholine (1 μM) constriction. N = 7 to 12 for controls (○); N = 4 to 5 for glibenclamide. For details, see the legend for figure 2.
What makes 4-AP a useful pharmacological tool for studying the KV channels is the fact that it does not inhibit the K Ca channels (Boyle et al., 1992) and potently inhibits the KV channels in millimolar concentrations. Hisada et al. (1990) have shown that 4-AP-sensitive currents may exist in guinea pig trachea. However, in the experimental conditions of this study, the 4-AP-sensitive channels did not play any role in the relaxing effects of the NO donors.

There is pharmacological evidence in the literature, in experiments using the K ATP channel openers cromakalim and lemakalim (BRL 38227), that K ATP channels exist in guinea pig trachea (Taylor et al., 1992). Glibenclamide can inhibit the smooth muscle mechano-inhibitory effects of the K 1 channel openers in vitro in guinea pig trachealis (Nielsen-Kudsk and Bang, 1991). In our experimental procedure, glibenclamide did not modify the relaxing effects of the NO donors used except that of GEA 3268. Glibenclamide did not significantly modify the effects of GEA 5145, although it is structurally similar to GEA 3268. This suggests that despite the significant effects of glibenclamide on GEA 3268 relaxation, the K ATP channels do not mediate NO-induced relaxation, which is in accordance with other findings in guinea pig trachea (Bialecki and Stinson-Fisher, 1995), though not in rabbit mesenteric arteries, in which NO has been reported to hyperpolarize through the K ATP channels (Murphy and Brayden, 1995). However, the number of the K ATP channels is reported to be much lower, only 300 to 500 channels per cell, whereas that of the K Ca channels is much higher, up to 10,000 per cell (Edwards and Weston, 1994). Therefore, the possibility of seeing any effects mediated by the K ATP channels is smaller. Also, the physiological control of this channel in many tissues may be primarily associated with other nucleotides, G proteins, various ligands or even pH (Edwards and Weston, 1993).

The K Ca channels have been identified in the airway smooth muscle of the guinea pig and several other species (Hisada et al., 1990; Kotlikoff, 1990). According to Galvez et al. (1990), both IbTX and ChTX bind at different sites on the K Ca channel and

<table>
<thead>
<tr>
<th>K+ Channel Inhibitors</th>
<th>A</th>
<th>Control</th>
<th>0.47 ± 0.07</th>
<th>0.50 ± 0.11</th>
<th>3.04 ± 0.47</th>
<th>2.64 ± 0.58</th>
</tr>
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<tr>
<td></td>
<td>4-AP</td>
<td>100 µM</td>
<td>0.21 ± 0.01*</td>
<td>0.41 ± 0.05</td>
<td>7.96 ± 0.20*</td>
<td>3.84 ± 1.82</td>
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<tr>
<td></td>
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<td>1 mM</td>
<td>0.59 ± 0.06</td>
<td>0.48 ± 0.01</td>
<td>6.40 ± 1.94</td>
<td>4.61 ± 1.75</td>
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<td>Glibenclamide 33 µM</td>
<td>0.39 ± 0.05</td>
<td>0.62 ± 0.15</td>
<td>3.97 ± 0.75</td>
<td>3.86 ± 1.24</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>K+ Channel Inhibitors</th>
<th>B</th>
<th>Control</th>
<th>1.55 ± 0.34</th>
<th>1.06 ± 0.18</th>
<th>13.2 ± 1.87</th>
<th>43.2 ± 12.1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4-AP</td>
<td>100 µM</td>
<td>1.07 ± 0.23</td>
<td>1.06 ± 0.24</td>
<td>13.1 ± 3.96</td>
<td>23.3 ± 8.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 mM</td>
<td>1.37 ± 0.29</td>
<td>1.10 ± 0.41</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glibenclamide 33 µM</td>
<td>0.98 ± 0.29</td>
<td>0.48 ± 0.08</td>
<td>11.8 ± 3.49</td>
<td>18.1 ± 5.21</td>
</tr>
</tbody>
</table>
modulate the activity by different mechanisms. ChTX is known to inhibit at least three different types of voltage-dependent K⁺ channels (Hermann and Erleben, 1989; Vázquez et al., 1990), but IbTX selectively inhibits the KCa channels without having any inhibitory effect on the other K⁺ channels affected nonselectively by ChTX (Candia et al., 1992).

In the present study, after metacholine constriction, IbTX inhibited significantly the relaxing effect of all the NO donors studied. ChTX inhibited the relaxations induced by all the other NO donors (but nonsignificantly those induced by SIN-1). When the inhibitory effects of these toxins are compared, IbTX is found to inhibit all the NO donors in metacholine constriction more than ChTX, which shows that IbTX is more potent than ChTX.

KCl depolarizes the cell to approximately –30 to –20 mV. At the same time, there is an increase in cytosolic calcium concentration that leads to contraction. With higher KCln concentrations, the probability of the KCa channels being closed would remain high, leading to decreased relaxation. Hamaguchi et al. (1992) showed that in KCN-constricted bovine trachea, 30 or 100 nM ChTX only slightly inhibited glyceryl trinitrate and sodium nitroprusside relaxation. Similarly, in this study when 40 mM KCl was used to induce constriction, the relaxations to all the NO donors were affected only slightly by ChTX, but the relaxations to SIN-1 and SNAP were significantly affected by IbTX. Therefore, the fact that the inhibiting effects of ChTX were less than those of IbTX on NO donor-induced relaxation suggests that IbTX is more potent for the KCa channels than is ChTX, independently of the constricting agents. In the presence of IbTX (100 nM), all the NO donors relax up to 20% in KCln constriction, which suggests that other relaxing mechanisms must be involved. In the case of GEA 3268 and GEA 5145, the toxins used did not modify the relaxing effect, which suggests that in KCln constriction, the relaxing effect does not involve the KCa channels.

In conclusion, the relaxing effects of NO-releasing drugs, independent of their structures, in guinea pig trachea in vitro, are partly mediated through the KCa channels and can be antagonized by the specific inhibitor of sGC, ODQ. Under these conditions, the KCATP channel inhibitor glibenclamide could not modify the relaxing effects of the NO donors studied, and the 4-AP-affected K⁺ channels played no role.

Acknowledgments

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References