Effects of IBMX on norepinephrine-induced vasoconstriction in small mesenteric arteries

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Taylor, Mark S., Hong Gao, Jason D. Gardner, and Joseph N. Benoit. Effects of IBMX on norepinephrine-induced vasoconstriction in small mesenteric arteries. Am. J. Physiol. 276 (Gastrointest. Liver Physiol. 39): G909–G914, 1999.—The present study assesses the effects of the phosphodiesterase inhibitor IBMX on norepinephrine (NE)-induced constriction of small mesenteric arteries. Arteries (∼150 µm) were dissected from rats and mounted on a wire myograph for isometric force measurement. NE concentration effect curves were generated after exposure to 500 µM IBMX for 60 min. IBMX significantly reduced NE-induced tension development. Studies were also conducted following sarcoplasmic reticulum (SR) depletion (ryanodine, 10 µM) or L-type Ca2+-channel blockade ([+]BAY K 8644, 10 µM) in the presence and absence of IBMX. Both SR depletion and L-channel blockade reduced NE-induced tension generation, consistent with incomplete Ca2+-mobilization. IBMX significantly attenuated NE responses in ryanodine and [-]BAY K 8644-treated vessels. Finally, treatment of NE-stimulated vessels with IBMX (500 µM) caused a reduction in vascular tension that was greater than the concomitant reduction in cytosolic Ca2+ concentration ([Ca2+]c), indicating that a portion of the IBMX-mediated relaxation is Ca2+-independent. These data suggest that IBMX attenuation of NE responsiveness not only involves a reduction in [Ca2+]c but also a significant decrease in Ca2+ sensitivity.

VASCULAR TONE IS DETERMINED by the simultaneous influences of constricting and dilating stimuli on vascular smooth muscle (VSM). Classically, vasoconstrictors such as norepinephrine (NE) are believed to trigger the release of Ca2+ from the sarcoplasmic reticulum (SR) and promote the influx of Ca2+ from the extracellular compartment (1, 9, 22). Elevated cytosolic Ca2+ concentration ([Ca2+]c) leads to the calmodulin-dependent activation of myosin light chain kinase, the phosphorylation of myosin, cross-bridge formation, and force development. It has also been suggested that NE may activate protein kinase C isoforms that can cause tension development independent of Ca2+ mobilization (9, 22). Reductions in vascular tone can result when the cellular mechanisms of smooth muscle contraction are opposed. Typically, vasorelaxation is mediated by elevations in VSM cyclic nucleotide (cAMP and cGMP) concentrations. Although cyclic nucleotides manifest their effects through stimulation of cyclic nucleotide-dependent protein kinases A and G (PKA and PKG), the specific cellular mechanisms that oppose VSM contraction remain unclear. Termination of cyclic nucleotide-dependent events involves the degradation of cAMP and cGMP by cellular phosphodiesterases (PDEs). PDE activity and regulation may ultimately determine cytosolic cyclic nucleotide concentrations, but the role of PDEs in the control of vascular tone is not well defined. Previous studies suggest that cyclic nucleotides principally limit smooth muscle contraction by lowering [Ca2+]c (16, 19). It has been postulated that the reduction in [Ca2+]c results from diminished influx of Ca2+ across the plasma membrane or altered Ca2+ handling by the SR (i.e., impaired Ca2+ release or increased Ca2+-sequestration). Furthermore, it has been proposed that cyclic nucleotides can reduce vascular tension through a mechanism that is independent of [Ca2+]c regulation (i.e., reduced myofilament Ca2+ sensitivity). Despite recent insights into cyclic nucleotide control of vascular tone, the principal mechanism responsible for the regulation of contraction is still unknown. In this study, we assess the effects of the nonspecific PDE inhibitor IBMX on NE-induced isometric tension generation in small mesenteric arteries. We also investigate possible mechanisms of IBMX-mediated attenuation of NE responses, including diminished Ca2+ influx from the extracellular compartment, altered Ca2+ release from the SR, and reduced Ca2+ sensitivity.

METHODS

Two segments of small mesenteric artery (∼1 mm in length and 150–200 µm internal diameter) were dissected from male Sprague-Dawley rats, bathed in pH-adjusted physiological salt solution (PSS) containing (in mM) 120 NaCl, 5 KCl, 2.5 CaCl2, 1.2 MgSO4, 25 NaHCO3, 1.2 NaH2PO4, 0.025 EDTA, and 5.5 glucose, and mounted on a wire myograph for isometric force measurement (20). Vessels were normalized by stretching to 90% of the internal circumference at an internal pressure of 100 mmHg (0.9 × IC100) as described by Mulvany and Halpern (20). This preset the internal vessel circumference for maximal active tension development. The vessels were allowed to equilibrate at 37°C for 60 min. At least two priming doses (10–4 M) of NE were applied to the vessels to ensure that maximal responses were reproducible. After washing, one of three protocols was followed. In the first protocol, vessels were exposed to 500 µM IBMX for 60 min before concentration-dependent responses to NE were assessed. In the second protocol, vessels were exposed to 10 µM ryanodine or treated with a combination of ryanodine (10 µM) and IBMX (500 µM) for 60 min before NE challenge. Ryanodine at this concentration is known to inhibit the SR-dependent component of NE-mediated VSM contraction (10). In the third protocol, vessels were treated with 10 µM (+)-BAY K 8644 or exposed to a combination of (+)-BAY K...
8644 (10 µM) and IBMX (500 µM) before NE challenge. At a concentration of 10 µM (+)-BAY K 8644 is an effective L-type Ca²⁺ channel blocker. It has been shown to completely relax KCl-constricted rabbit aorta (5) and maximally inhibit NE constriction in rat portal vein (7). For each protocol, a separate group of vessels was treated with vehicle (0.1% DMSO) for 60 min before NE challenge. These vessels served as controls. For all concentration-effect data, isometric force (mN/mm) was normalized for vessel length and basal tone, yielding the net change in tension per millimeter vessel (ΔmN/mm). IBMX, ryanodine, and (+)BAY K 8644 were purchased from Research Biochemicals International (Natick, MA).

In a final series of experiments, simultaneous measurements of [Ca²⁺]i and tension generation were obtained. Arteries were mounted on the myograph and normalized as previously described. The vessels were then incubated with the acetoxymethyl ester of the fluorescent Ca²⁺-indicator indo 1 for 90 min. The loading solution contained indo 1 (10 µM), DMSO (0.4%), Pluronic F-127 (0.1%), and Cremophor (0.2%). The arteries were washed with normal PSS and left undisturbed for 30 min before the myograph was mounted on the stage of an inverted, photometer-equipped microscope. The microscope was focused on the smooth muscle layer of the artery. Vessels were illuminated with monochromatic light (350 nm), and emitted light was detected at wavelengths of 405 and 485 nm using photomultiplier tubes. The signals from the photomultipliers and force transducer were digitized and stored on computer to yield simultaneous recordings of Ca²⁺-dependent fluorescence and force generation. Force values were subsequently divided by vessel length and expressed as tension (mN/mm). Vessels in these experiments were preconstricted with NE (10⁻⁵ M) and subsequently exposed to IBMX (500 µM) or nifedipine (10 µM).

Data analysis. Responses were obtained from two vessels from the same animal, averaged, and counted as a single observation. The data presented depict five to six observations per protocol. Data are expressed as means ± SE. The pD₂ (−log[EC₅₀]) was calculated using nonlinear regression analysis of the concentration-effect curves. One-way ANOVA was used to identify statistical trends in the data set. When appropriate, a Bonferroni post hoc analysis was employed to distinguish statistical differences between groups. An asterisk (*) indicates significance between control and treated groups and a pound sign (#) indicates significance between two treated groups. P < 0.05 was considered significant. For simultaneous [Ca²⁺]i and tension measurements, the ratio of F₄₀₅/F₄₈₅ is used as an index of [Ca²⁺]i and tension is reported in millinewtons per millimeter.

RESULTS

Effects of IBMX on NE-induced tension generation. NE-induced constriction was attenuated in vessels pretreated with 500 µM IBMX, as evidenced by a right, downward shift in the NE concentration-effect curve (Fig. 1). Maximal tension generation was significantly reduced from 8.61 ± 0.99 mN/mm in control vessels to 1.93 ± 0.14 mN/mm in IBMX-treated vessels (P < 0.05). Vascular NE sensitivity was also reduced as evidenced by a decrease in pD₂ values from 5.95 ± 0.10 in control to 5.32 ± 0.15 in IBMX-treated vessels (P < 0.05). IBMX had no effect on resting basal tension.

Effects of IBMX on NE-induced tension generation after inhibition of internal Ca²⁺ store release with ryanodine. In the second group of experiments (Fig. 2), maximal NE-induced tension in control vessels reached 5.79 ± 0.92 mN/mm. In vessels treated with ryanodine (10 µM), maximal tension was significantly reduced (2.60 ± 0.85 mN/mm) compared with controls (P < 0.05). Vessels treated with a combination of ryanodine (10 µM) and IBMX (500 µM) showed a further reduction in maximal tension development (0.20 ± 0.03 mN/mm) that was significantly different from both controls and IBMX alone.

![Fig. 1. Effects of IBMX treatment on norepinephrine (NE) responses in small mesenteric arteries. Vessel segments were mounted on a small wire myograph and exposed to IBMX (500 µM) for 60 min (■) or treated with vehicle (0.1% DMSO) alone for 60 min (○) before NE concentration effect curve was generated. Each response represents change from basal tension and is expressed as means ± SE. *Significant difference from control (P < 0.05). pD₂ = −log[EC₅₀].](http://ajpgi.physiology.org/doi/abs/10.1152/ajpheart.01107.2006)
control and ryanodine-treated groups (P < 0.05). The pD2 values showed a similar trend, decreasing from 6.09 ± 0.08 in control vessels to 5.82 ± 0.03 in vessels treated with ryanodine alone (P < 0.05). Vessels treated with both ryanodine and IBMX showed a further shift in pD2 to 5.41 ± 0.06 (P < 0.05 vs. control and P < 0.05 vs. ryanodine). Thus IBMX reduced maximal tension development and NE potency to a greater degree than that achieved with ryanodine alone.

Effects of IBMX on NE-induced tension generation in the presence of the Ca2+ channel blocker (+)-BAY K 8644. In the third group of experiments (Fig. 3), control vessels yielded maximal tension development of 5.71 ± 0.38 mN/mm, which was significantly reduced to 2.25 ± 0.60 mN/mm in vessels treated with 10 µM of the Ca2+ channel blocker (+)-BAY K 8644 (P < 0.05). A further reduction in maximal tension (0.72 ± 0.06 mN/mm) was observed in vessels exposed to a combination of (+)-BAY K 8644 (10 µM) and IBMX (500 µM), which was significantly different from control and (+)-BAY K 8644-treated groups (P < 0.05). Again, pD2 values mimicked maximal tension responses, decreasing from 5.97 ± 0.11 in control vessels to 5.56 ± 0.05 in those treated with (+)-BAY K 8644 alone (P < 0.05). The pD2 value diminished to 5.02 ± 0.13 in vessels treated with a combination of (+)-BAY K 8644 and IBMX (P < 0.05 vs. control and P < 0.05 vs. (+)-BAY K 8644). Therefore, IBMX enhanced the reduction in maximal tension generation and NE sensitivity caused by the Ca2+ channel blocker (+)-BAY K 8644. Table 1 summarizes maximal tension and pD2 values for these experiments.

Effects of IBMX on [Ca2+]i and tension in NE-stimulated arteries. Figure 4, top, depicts the effects of IBMX on [Ca2+]i (F405/F485) and tension in a NE-stimulated small mesenteric artery. The vehicle DMSO (0.1%) had no effect on fluorescence or tension. NE produced a rapid increase in [Ca2+]i, which preceded the rise in tension. Sustained plateaus in both [Ca2+]i and tension were observed in the control and IBMX-treated groups (P < 0.05). The pD2 value diminished to 5.02 ± 0.13 in vessels treated with a combination of (+)-BAY K 8644 and IBMX (P < 0.05 vs. control and P < 0.05 vs. (+)-BAY K 8644). Table 1 summarizes maximal tension and pD2 values for these experiments.

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**Table 1. Summary of IBMX, ryanodine, and (+)-BAY K 8644 effects on norepinephrine concentration-effect curves in small mesenteric arteries**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximal Tension, mN/mm</th>
<th>pD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.61 ± 0.99</td>
<td>5.95 ± 0.10</td>
</tr>
<tr>
<td>IBMX</td>
<td>1.93 ± 0.14*</td>
<td>5.32 ± 0.15*</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>5.79 ± 0.92</td>
<td>6.09 ± 0.08</td>
</tr>
<tr>
<td>Ryanodine + IBMX</td>
<td>2.60 ± 0.85*</td>
<td>5.82 ± 0.03*</td>
</tr>
<tr>
<td>Control + IBMX</td>
<td>0.20 ± 0.03†</td>
<td>5.41 ± 0.06†</td>
</tr>
<tr>
<td>(+)-BAY K 8644</td>
<td>5.71 ± 0.38</td>
<td>5.97 ± 0.11</td>
</tr>
<tr>
<td>(+)-BAY K 8644 + IBMX</td>
<td>2.25 ± 0.60*</td>
<td>5.56 ± 0.05*</td>
</tr>
<tr>
<td></td>
<td>0.72 ± 0.06†</td>
<td>5.02 ± 0.13†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5–6 observations/protocol. *Significant difference (P < 0.05) from control responses. †Significant difference (P < 0.05) from responses in presence of ryanodine or (+)-BAY K 8644 alone. pD2 = -log(EC50).

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**Fig. 3. Effects of (+)-BAY K 8644 or combination of (+)-BAY K 8644 and IBMX on NE responses in small mesenteric arteries.** Vessel segments were mounted on small wire myograph and exposed to 10 µM (+)-BAY K 8644 (○), 10 µM (+)-BAY K 8644 + 500 µM IBMX (△), or treated with vehicle (0.1% DMSO, ■) alone for 60 min before NE concentration effect curve was generated. Each response represents change from basal tension and is expressed as means ± SE. *Significant difference from control (P < 0.05). †Significant difference from (+)-BAY K 8644 (P < 0.05).

**Fig. 4. Simultaneous recordings of cytosolic Ca2+ concentration ([Ca2+]i; F405/F485) and tension showing effects of IBMX or nifedipine on NE-stimulated small mesenteric artery segment.** Artery segments were mounted on wire myograph and stimulated with 10−5 M NE. Once [Ca2+]i and tension reached plateau levels, the vessels were exposed to 500 µM IBMX (top) or 10 µM nifedipine (Nif, bottom). Note that, in IBMX-treated vessel, biphasic reduction in [Ca2+]i is associated with relatively steady decline in vascular tension, whereas [Ca2+]i and tension decrease proportionally after nifedipine treatment.

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and tension were subsequently achieved. Addition of IBMX (500 µM) to the bath initially caused a rapid reduction in [Ca\textsuperscript{2+}]i and tension. Thereafter, the decrease in tension was disproportionately larger than the decrease in the Ca\textsuperscript{2+} signal. For comparison, a separate group of vessels was stimulated with NE as described and subsequently exposed to the Ca\textsuperscript{2+} channel blocker nifedipine (10 µM). In this case, tension and [Ca\textsuperscript{2+}]i decreased proportionally throughout the relaxation (Fig. 4, bottom).

**DISCUSSION**

PDE inhibition in VSM elicits vasorelaxation by preventing the degradation of and thereby elevation of intracellular cyclic nucleotides (13, 24). Both cAMP-degrading (PDE3 and PDE4) and cGMP-degrading (PDE1 and PDE5) PDE isozymes have been identified in VSM (12, 18). In studies of rat aorta by Lugnier et al. (18), the nonspecific PDE inhibitor IBMX at 500 µM elevated both cAMP and cGMP levels approximately sevenfold. This concentration of IBMX has also been found to effectively relax PGF\textsubscript{2α}-contracted, endothelium-denuded pulmonary artery rings (26). IBMX was used in the present study to assess the global effects of PDE inhibition on vasoconstrictor responses in mesenteric resistance arteries.

It has been suggested that the inhibitory effects of cAMP and cGMP may be mediated by PKA- and/or PKG-dependent phosphorylation of membrane ion channels (2, 15, 21, 29, 34, 35). Conductance through L-type Ca\textsuperscript{2+} channels is reduced with cyclic nucleotide elevation and PKA activation (34). In addition, PKA-mediated increases in K+ conductance have been reported in VSM cell membranes (21, 29, 35). Whether the inhibition of Ca\textsuperscript{2+} influx derives directly from L-type Ca\textsuperscript{2+} channel regulation or indirectly via membrane hyperpolarization is still unknown. The inability of VSM L-type Ca\textsuperscript{2+} channels to open or remain open during vasoconstrictor receptor stimulation would limit [Ca\textsuperscript{2+}]i and hence attenuate the onset and maintenance of contraction. In addition to the documented action on membrane ion channels, cyclic nucleotides have also been reported to modulate Ca\textsuperscript{2+} release and sequestration, an action that is linked to protein kinase-dependent phosphorylation of intracellular targets. Although biochemical evidence of cyclic nucleotide-dependent phosphorylation of inositol 1,4,5-triphosphate receptors (3, 4, 28) exists, evidence of such regulation in intact VSM preparations is lacking. PKA enhancement of Ca\textsuperscript{2+}-ATPase activity at the SR has been reported (6, 11, 32) to result from disinhibition of the regulatory protein phospholamban (27). To this end, activation of phospholamban would augment Ca\textsuperscript{2+} uptake and oppose vasoconstrictor-induced elevations in [Ca\textsuperscript{2+}]i. Despite the fact that potential sites of cyclic nucleotide-dependent Ca\textsuperscript{2+} regulation have been identified, the relative contribution of each of these pathways in the maintenance of smooth muscle contractile tension remains uncertain.

The results of the present study reveal substantial depression of maximal tension and loss of NE sensitivity in mesenteric vessels treated with 500 µM IBMX. Thus PDE inhibition can effectively attenuate vasoconstrictor responses in resistance arteries. These findings suggest that regulation of PDE activity represents a potentially important physiological means of influencing vascular tone. The importance of such physiological regulation becomes apparent if one considers the factors governing PDE isof orm activity in VSM cells. For example, it has been suggested that inhibition of the cAMP-specific type 3 PDE (PDE3) by endogenous cGMP relaxes VSM through elevation of cAMP (16, 25). However, to our knowledge, direct evidence of this permissive action of cGMP on VSM tension does not exist.

Our studies further evaluate possible cellular mechanisms of IBMX effects on tension generation. Studies by others have clearly documented the importance of ryanodine-sensitive Ca\textsuperscript{2+} stores to the early phase of NE-induced tension development in VSM (9, 10). Our data show that ryanodine alone significantly reduces NE sensitivity and maximal tension. Under these conditions, VSM is predominantly dependent on extracellular Ca\textsuperscript{2+} for activation of the contractile elements. Addition of IBMX to ryanodine-treated arteries further attenuated NE responses. In fact, tension generation was almost completely abolished. From this series of experiments, we conclude that tension generation related to Ca\textsuperscript{2+} influx is attenuated by IBMX.

In the next series of experiments, we examined the effects of IBMX on tension predominantly related to release of Ca\textsuperscript{2+} from the SR. To minimize the effects of extracellular Ca\textsuperscript{2+}, we inhibited membrane L-type channels with the dihydropyridine (+)-BAY K 8644. Under these conditions, tension generation is largely dependent on SR Ca\textsuperscript{2+} stores. We observed that IBMX also attenuated SR-dependent NE responses. In view of these findings we conclude that it is unlikely that the effects of cyclic nucleotides are limited to impairment of voltage-sensitive Ca\textsuperscript{2+} influx alone.

Collectively, the ryanodine and (+)-BAY K 8644 experiments suggest that IBMX attenuates vasoconstrictor responses in vessels limited to Ca\textsuperscript{2+} recruitment from intracellular or extracellular sources. Such findings could be interpreted either as alteration in multiple Ca\textsuperscript{2+} mobilization pathways or alteration in Ca\textsuperscript{2+}-dependent contractile events (i.e., Ca\textsuperscript{2+} sensitivity). To further examine the mechanism of IBMX action on tension development, we simultaneously monitored [Ca\textsuperscript{2+}]i and tension in mesenteric arterial preparations (Fig. 4). Within 30 s, IBMX produced a rapid reduction in [Ca\textsuperscript{2+}] and a corresponding decrease in contractile tension in NE-stimulated vessels. The rapid phase was followed by a slow phase of relaxation in which tension continued to fall with relatively little change in [Ca\textsuperscript{2+}]i. A separate group of vessels was treated with nifedipine following NE stimulation. Nifedipine relaxation is entirely Ca\textsuperscript{2+}-dependent, reducing vessel contraction by restricting Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels. The relationship between [Ca\textsuperscript{2+}]i and tension is illustrated in Fig. 5. Point A represents the [Ca\textsuperscript{2+}]i and corresponding steady-state tension resulting from NE...
Fig. 5. Plot of \([\text{Ca}^{2+}]\) vs. tension for IBMX-induced relaxation of NE-stimulated artery segments. Points represent \([\text{Ca}^{2+}]\) and tension values at the end of the rapid phase of IBMX relaxation. Point C represents \([\text{Ca}^{2+}]\) and tension values 150 s after addition of IBMX. Nifedipine-induced relaxation is also depicted in Fig. 5. The slope (m = 1.06 ± 0.02) of the line representing nifedipine relaxation (A→D) was not different from the line of identity (m = 1.00), illustrating a direct one-to-one relationship between \([\text{Ca}^{2+}]\) and tension. Segment A→B represents the changes in \([\text{Ca}^{2+}]\) and tension immediately following IBMX exposure. The slope (m = 0.97 ± 0.10) of this segment was not statistically different from that of nifedipine (A→D), indicating that the early phase of relaxation was totally dependent on \([\text{Ca}^{2+}]\) removal. Segment B→C illustrates the changes in \([\text{Ca}^{2+}]\) and tension during the slow phase of IBMX relaxation. The slope (m = 2.00 ± 0.38) of this segment was significantly different (P < 0.05) from that of nifedipine (A→D). We interpret this finding to indicate that vascular tension becomes less dependent on \([\text{Ca}^{2+}]\) levels during the latter stages of IBMX-induced relaxation. This observation is consistent with the contention that cyclic nucleotide-induced relaxation of VSM results at least in part from a reduction in \([\text{Ca}^{2+}]\) sensitivity of the contractile apparatus.

Evidence of cyclic nucleotide-dependent reduction in myofilament \([\text{Ca}^{2+}]\) sensitivity has been previously reported. Nishimura and van Breemen (23) found that elevation of cAMP or cGMP levels relaxed \(\alpha\)-toxin-permeabilized smooth muscle contracted at constant \([\text{Ca}^{2+}]\). The mechanism of \([\text{Ca}^{2+}]\) desensitization is unclear but may involve inhibition of myosin light chain kinase (31) and/or activation of myosin light chain phosphatase (MLCP) by cyclic nucleotide-dependent protein kinases (14, 30). Recently, it was suggested that cyclic nucleotides may recruit MLCP from a membrane-associated inactive state to a cytosolic active state (8). It has also been proposed that cyclic nucleotides enhance the activity of the regulatory protein telokin, which normally activates MLCP (33). Presently, evidence linking cyclic nucleotides directly to \([\text{Ca}^{2+}]\)-independent changes in myosin light chain phosphorylation in VSM is limited. Future investigation into the role of MLCP regulation is warranted.

In summary, the present study reveals that the nonspecific PDE inhibitor IBMX reduces NE-induced tension generation in small mesenteric arteries. Simultaneous measurement of \([\text{Ca}^{2+}]\) and contractile tension further suggests that the vasorelaxation induced by IBMX results from a reduction in VSM \([\text{Ca}^{2+}]\), as well as a reduction in \([\text{Ca}^{2+}]\) sensitivity. These findings suggest that cyclic nucleotide-mediated attenuation of agonist-induced arterial constriction does not result entirely from the regulation of any single pathway of \([\text{Ca}^{2+}]\) mobilization. Additional studies are necessary to fully understand the cellular mechanism(s) of cAMP-
and cGMP-mediated attenuation of vasoconstrictor ef-

cffectiveness.

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