Pharmacological profile of depressor response elicited by sarthran in rat ventrolateral medulla

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Ito, Satoru, and Alan F. Sved. Pharmacological profile of depressor response elicited by sarthran in rat ventrolateral medulla. Am J Physiol Heart Circ Physiol 279: H2961–H2966, 2000.—Injection of sarthran, an angiotensin receptor antagonist, bilaterally into the rostral ventrolateral medulla (RVLM) of α-chloralose-anesthetized rats decreases arterial pressure (AP) to the same extent as total autonomic blockade. This response is not reproduced by selective AT1 antagonists. To examine the pharmacological profile of the response elicited by [Sar1, Thr8]ANG II (sarthran), the ability of angiotensin analogs to inhibit the effect of sarthran injected into the RVLM was tested. Coinjection of angiotensin II (ANG II) prevented the sarthran-evoked decrease in AP, but this action of ANG II was markedly attenuated by pre-treatment of the RVLM with the aminopeptidase inhibitor amastatin. Coinjection of ANG(3–8) or a selective agonist of AT1 receptors prevented the effect of sarthran injected into the RVLM. ANG(1–7) was also able to prevent the effect of sarthran. None of the angiotensin fragments tested substantially altered blood pressure when injected alone into the RVLM. These results suggest that the depressor action of sarthran injected into the RVLM is not dependent on ANG II receptors, though the nature of the site or sites of action of sarthran within the RVLM remains uncertain.

angiotensin; angiotensin antagonist; angiotensin AT4 receptor; neural control of blood pressure

The rostral ventrolateral medulla (RVLM) appears to be the crucial supraspinal site involved in the tonic maintenance of arterial blood pressure (AP) in anesthetized animals (5). Thus inhibition or destruction of the RVLM causes AP to decrease to the same extent as does transection of the cervical spinal cord or autonomic ganglionic blockade. We have reported that bilateral injection into the RVLM of the angiotensin (ANG) receptor antagonists [Sar1, Thr8]ANG II (sarthran) or [Sar1, Ile8]ANG II (sarile) also cause AP to decrease to the same extent as autonomic blockade (15), suggesting that a tonically active sarthran-sensitive input to RVLM sympathoexcitatory neurons plays a prominent role in the maintenance of resting AP in anesthetized rats. Other laboratories have confirmed this observation (13, 32) and further demonstrated that the decrease in AP is accompanied by a large decrease in sympathetic nerve activity.

METHODS

These experiments were conducted on adult male Sprague-Dawley rats (Zivic-Miller, Allison Park, PA, or Charles River, Yokohama, Japan) weighing 280–400 g. The rats were housed singly in wire-mesh cages in a temperature-controlled room on a 12:12-h light-dark cycle with food and tap water available ad libitum for at least 1 wk before use in experiments.

Rats were prepared for brain stem injections as previously described (15). Briefly, rats were anesthetized with halothane (2% in 100% O2 administered through a cone placed over the nose), and a cannula (polyethylene-50 tubing filled with heparinized saline) was inserted into the right femoral artery for recording of AP and heart rate (HR). A second cannula was placed in the right femoral vein for administering drugs. The trachea was cannulated, and rats were artificially ventilated with 2% halothane in 100% O2, followed by the administration of a muscle relaxant (d-tubocurarine, 0.5 mg/kg iv, supplemented hourly with 0.2 mg/kg iv; tubocurarine was administered as a 1 mg/ml solution in saline).

The rats were placed in a stereotaxic instrument (Kopf Instruments) with the incisor bar positioned 11 mm below the interaural line. The dorsal surface of the medulla was exposed by limited craniotomy and the area postrema visualized. α-Chloralose was administered (60 mg/kg iv, supplemented hourly with 20 mg/kg iv; α-chloralose was administered as a 12 mg/ml solution in warmed saline and infused at a rate of ~1 ml/min), and the halothane was terminated. The
rats were ventilated with 100% O₂ throughout the remainder of the experiment. After the surgical manipulations were completed, the rats were allowed to stabilize for at least 20 min before the start of the experiment. We injected drugs into the brainstem, as previously described (15), by using single-barrel glass micropipettes. All of the drugs, except CV-11974, were dissolved in artificial cerebrospinal fluid (aCSF, in mM: 144 NaCl, 1.2 CaCl₂, 2.8 KCl, and 0.9 MgCl₂) and injected in a 100-nl volume over a period of several seconds with the use of a PicoPump (WPI, New Haven, CT). CV-11974 was injected either in 10 mM bicarbonate in aCSF or in aCSF with pH increased to ~10 by the addition of NaOH. For bilateral injections, an injection was made on one side, the pipette was withdrawn from the brain and positioned on the contralateral side, and the contralateral injection was made; thus the two injections were made ~1 min apart.

Initially, microinjections of glutamate (1 nmol) were made into the medulla to establish coordinates for functional pressor sites in the left and right RVLM; a pressor response of at least 30 mmHg was taken as the minimal acceptable response. Coordinates for RVLM sites used in this study were, relative to the caudal tip of the area postrema and with the pipette angled 20° rostrally, 1.6–2.0 rostral, 1.7–2.1 mm lateral (almost always 1.9), and 2.6–3.2 mm ventral. After the sites were functionally identified, we began the experiments. The protocols of individual sets of experiments are presented along with the results.

At the conclusion of experiments in many rats, ~20 nl of 1% fast green was injected into the RVLM with the use of the same micropipette that was previously used for drug injections to verify the center of the injection site. The rats were then decapitated and the brain stems rapidly removed and frozen in isopentane on dry ice. The brain stems were subsequently cut into 40-μm sections by using a cryostat, and sections were mounted on glass microscope slides. Sections were stained with neutral red. Functionally identified pressor sites in the RVLM were always located within the RVLM, ~500 μm ventral to the compact portion of the nucleus ambiguus at the rostral-caudal plane corresponding to ~2.8 mm from the interaural point, on the basis of the atlas of Paxinos and Watson (23). We have previously published a photomicrograph of a typical RVLM microinjection site (15).

The following drugs were used in these studies: sarthran (Sigma Chemical, St. Louis, MO, and Bachem, Torrance, CA), ANG II (Sigma Chemical), ANG(3–8) (ANG IV), ANG(1–7), [7-D-Ala]ANG(1–7), ANG(3–7) (Bachem), norleucine-1-ANG IV (Nle-ANG IV), divalinal-ANG IV (16) supplied by Joseph Harding, University of Washington, Pulman, WA), amastatin (Sigma Chemical), muscimol (Research Biochemicals International, Wayland, MA), and chlorisondamine (generously donated by Ciba-Geigy, Summit, NJ). Other drugs and chemicals were obtained from standard commercial suppliers.

Data are expressed as means ± SE and were analyzed by Student’s t-test or ANOVA, followed by the Newman-Keuls test (Systat, Evanston, IL).

RESULTS

Effects of selective AT₁ receptor antagonists. As previously noted (15), bilateral injection of 1 nmol sarthran into the RVLM in α-chloralose-anesthetized rats reduced mean arterial pressure (MAP) from ~110 to ~60 mmHg (Table 1). In contrast, injection of neither losartan nor CV-11974, two selective nonpeptide AT₁ receptor antagonists, at a dose of 1 nmol mimicked this effect (Table 1). Lower doses of losartan and CV-11974 also did not decrease MAP (100 pmol of losartan, n = 3; 100, 200, and 500 pmol of CV-11974, 1 rat at each dose; data not shown).

Ability of angiotensin peptides to prevent the effects of sarthran. We have previously reported that the marked hypotensive effect of sarthran (but not the GABA receptor agonist muscimol) injected bilaterally into the RVLM could be prevented by the coinjection of 200 pmol of ANG II (15). As a first step in further characterizing this action of ANG II, the ability of lower doses of ANG II to prevent the effects of coinjected sarthran was tested. The lowest dose of ANG II that prevented the hypotensive effect of 1 nmol of sarthran was 200 pmol; 50 pmol of ANG II attenuated the sarthran-evoked decrease in MAP by ~50% (Fig. 1).

To determine whether this action of injected ANG II to prevent the hypotensive effect of sarthran required the metabolism of ANG II to some other compound, the effects of amastatin, an aminopeptidase inhibitor, on the effects of ANG II were examined. A bilateral injection of 1 nmol amastatin had little effect on resting MAP and HR. In seven rats receiving only bilateral injections of 1 nmol amastatin into the RVLM, baseline MAP was 101 ± 6 mmHg and transiently increased to 109 ± 6 mmHg after injection; HR decreased transiently from 344 ± 15 to 327 ± 10 beats/min. Within 5 min of the injection of amastatin, MAP and HR had returned to control levels. In rats receiving bilateral injections of amastatin ~7 min beforehand, injection of 1 nmol sarthran plus 200 pmol ANG II decreased MAP (Table 2), although not to the same extent as in amastatin-pretreated rats injected with sarthran alone. This attenuation of the effect of ANG II by amastatin suggested that fragments of ANG II with amino-terminal amino acids removed might be the active compounds. ANG (3–8) (i.e., ANG IV), like ANG II, reversed the effects of 1 nmol sarthran injected into the RVLM (Fig. 1). However, ANG IV was approximately four- to fivefold less potent than ANG II; 1 nmol was the smallest dose of ANG IV tested that completely prevented the decrease in MAP caused by sarthran. The effectiveness of 1 nmol ANG IV in reversing the

| Table 1. Effect of injection of AT₁ receptor antagonists into RVLM |
|---|---|---|---|
| | n | MAP, mmHg | Heart Rate, beats/min |
| | Baseline | Change | Baseline | Change |
| Losartan 4 | 111 ± 8 | 1 ± 10 | 310 ± 4 | 12 ± 8 |
| CV-11974 3 | 128 ± 3 | 32 ± 12* | 377 ± 32 | 6 ± 6 |
| Sarthran 6 | 111 ± 7 | −50 ± 8* | 340 ± 2 | −75 ± 10* |

Values are means ± SE, n = no. of rats. RVLM, rostral ventrolateral medulla; MAP, mean arterial pressure. Anesthetized rats received bilateral injections of losartan, CV-11974, or [Ser¹, Thr²]ANG II (sarthran) (1 nmol of each). Studies were conducted in rats anesthetized with α-chloralose, with the exception of 2 rats that received losartan that were anesthetized with urethan (1.5 g/kg iv).

*Significant change from preinjection baseline (P < 0.05). Response to sarthran is significantly different from the response to either of the other drugs.
not tested. In contrast to the ability of Nle-ANG IV to prevent the marked decrease in AP caused by injection of sarthran into the RVLM, Nle-ANG IV had no effect on the decrease in AP produced by injection of muscimol into the RVLM (Fig. 2). Our results with Nle-ANG IV prompted us to test a putative AT4 receptor antagonist, divalinal-ANG IV (16). Injection of 1 nmol divalinal-ANG IV bilaterally into the RVLM did not significantly decrease MAP (−1 ± 2 mmHg; n = 5) or HR (0 ± 0 beats/min) (n = 4). Interestingly, when 1 nmol of sarthran was injected 5 min later, it did not cause a decrease in MAP (−5 ± 2 mmHg). Nonetheless, pretreatment with divalinal-ANG IV did not prevent the decrease in MAP caused by injection of 200 pmol of muscimol into the RVLM (95 ± 4 mmHg before muscimol vs. 69 ± 2 mmHg 3 min after muscimol, n = 4).

Fig. 1. Effects of angiotensin II (ANG II) and angiotensin IV (ANG IV) on the depressor response evoked by injection of sarthran into the rostral ventrolateral medulla (RVLM). Groups of α-chloralose-anesthetized rats received bilateral injections into the RVLM of [Sar1, Thr8]ANG II (sarthran, 1 nmol) or sarthran mixed with one of the doses of either ANG II or ANG IV (n = 4–6 per dose). In 21 rats used for the ANG II dose-response curve, baseline mean arterial pressure (MAP) and heart rate (HR) were 109 ± 2 mmHg and 349 ± 8 beats/min, respectively. In 14 rats used in the ANG IV dose-response curve, baseline MAP and HR were 109 ± 2 mmHg and 327 ± 9 beats/min, respectively. The dose-response data for ANG II and ANG IV preventing sarthran-evoked bradycardia were similar to those for the depressor response (data not shown). *Significant difference from the next lower dose of ANG peptide, P < 0.05. †Significant difference from injection value.

Table 2. Effect of amastatin pretreatment on the effect of ANG II and ANG IV on sarthran-evoked decreases in blood pressure

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>MAP, mmHg</th>
<th>Heart Rate, beats/min</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Baseline</td>
<td>Change</td>
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<tr>
<td>Sarthran</td>
<td>4</td>
<td>119 ± 8</td>
<td>−49 ± 8</td>
</tr>
<tr>
<td>Sarthran + ANG II</td>
<td>4</td>
<td>126 ± 4</td>
<td>−36 ± 1 †</td>
</tr>
<tr>
<td>Sarthran + ANG IV</td>
<td>5</td>
<td>126 ± 2</td>
<td>−5 ± 5 †</td>
</tr>
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</table>

Values are means ± SE, n = no. of rats. α-Chloralose-anesthetized rats were pretreated with bilateral injections of 1 nmol amastatin into the RVLM. Approximately 7 min later, 1 nmol sarthran or 1 nmol sarthran + 200 pmol ANG II or 1 nmol sarthran + 1 nmol ANG IV was injected bilaterally into the RVLM. Baseline values refer to the values just before sarthran injections (i.e., after amastatin), and the change in MAP and heart rate was recorded from this baseline at 3 min postinjection of sarthran. *Significant change from baseline, P < 0.05. †Significant difference from the response to injection with sarthran, P < 0.05. Response to sarthran in amastatin-pretreated rats was not statistically different from the response to sarthran (shown in Table 1).

Fig. 2. Effects of norleucine (Nle)-ANG IV on the depressor response evoked by injection of sarthran or muscimol into the RVLM. α-Chloralose-anesthetized rats received bilateral injections into the RVLM of either sarthran (n = 7), sarthran plus Nle-ANG IV (1 nmol, n = 8), muscimol (n = 4), or muscimol plus Nle-ANG IV (n = 5). Doses used were 1 nmol of sarthran, 200 pmol of Nle-ANG IV, and 200 pmol of muscimol. Data presented represent the maximal decrease in MAP that occurred within 5 min after injection. Baseline MAP and HR values for the rats used in this experiment were 111 ± 3 mmHg and 370 ± 4 beats/min, respectively. *Significant difference from all other treatment groups, P < 0.05.
When sarthran was injected 5 min later, it reduced produced by [Sar1]-ANG II was not different from that substantial decrease in MAP. The initial increase in MAP, followed several minutes later by a dose-response data for ANG(1–7) preventing sarthran-evoked bradycardia were similar to those for the depressor response (data not shown). *Significant difference from the next lower dose of ANG peptide, P < 0.05. $Lack of significant difference from preinjection value.

Because the emerging pharmacology of the marked depressor response caused by injection of sarthran (or sarile) into the RVLM appeared distinct from responses mediated by known angiotensin receptors, two additional compounds were tested to see if the response was specifically related to the sarcosine present in the one position of these compounds. [Sar1]-ANG II, a long-acting agonist at angiotensin receptors, injected into the RVLM (1 nmol, n = 4) produced a small increase in MAP, followed several minutes later by a substantial decrease in MAP. The initial increase in MAP was 9 ± 4 mmHg and lasted for 1.8 ± 0.6 min. This was followed by a sustained decrease in MAP of 45 ± 3 mmHg. The magnitude of the decrease in MAP produced by [Sar1]-ANG II was not different from that caused by sarthran. We also tested a [Sar1]-containing peptide unrelated to angiotensin (Sar-Arg-Gly-Asp-Pro, a sarcosine-containing fragment of fibronectin). Injection of 1 nmol of this peptide bilaterally into the RVLM had no effect on MAP (+3 ± 2 mmHg, n = 5). When sarthran was injected 5 min later, it reduced MAP by 31 ± 2 mmHg.

**DISCUSSION**

We have previously reported that injection of sarthran or sarile, peptide inhibitors of ANG receptors (8, 20), into the RVLM decreases MAP to the same extent as total inhibition of the RVLM (15). The goal of the present study was to further characterize the pharmacological profile of this response. The key finding of this study is that the ability of angiotensin-like peptides to reverse the sarthran-evoked decrease in MAP suggests an action on a receptor that is distinct from any previously characterized angiotensin receptor.

It is now well documented that injection of sarthran into to the RVLM decreases AP and sympathetic nerve activity. Initial studies found that unilateral injections of sarthran into the RVLM in several species (3, 4, 22, 26), including conscious rats (6), elicit a decrease in AP. Bilateral injections of sarthran or sarile into the RVLM of anesthetized rats produces a marked decrease in AP (13, 15, 32) similar to the decrease in AP produced by complete inhibition of the RVLM (15). The decrease in AP caused by injection of sarthran into the RVLM is accompanied by a decrease in sympathetic nerve activity (32). Curiously, one study (18) failed to observe this profound decrease in AP in response to injection of sarthran into the RVLM. The reason that Lin et al. (18) did not observe this response is unclear; although it is the only published report that used pentobarbital-anesthetized rats, we have found that bilateral injections of sarthran (1 nmol) into the RVLM of rats anesthetized with pentobarbital (50 mg/kg ip) reduced MAP by 42 ± 4 mmHg (n = 4; unpublished observation), which is similar to what we have reported in rats anesthetized with either α-chloralose or urethan.

The observation that neither losartan nor CV11974, two selective nonpeptide AT1 receptor antagonists, caused a decrease in MAP when injected bilaterally into the RVLM is consistent with previous reports (4, 6, 14, 31). For example, Averill et al. (4) noted that unilateral injection of losartan into the RVLM of halothane-anesthetized rats did not reduce MAP and at doses of 1 nmol or higher actually increased MAP. This lack of a depressor response caused by injection of losartan contrasted with a decrease in MAP of -25 mmHg caused by sarthran in that study (4). Thus it seems clear that sarthran injected into the RVLM does not reduce baseline MAP by selectively blocking the AT1 receptor.

In an effort to characterize the type of receptor in the RVLM on which sarthran acts to elicit a decrease in MAP, we examined the ability of ANG peptides to reverse the effects of sarthran. In contrast to studies in other laboratories examining the actions of ANG injected into the RVLM on cardiovascular regulation, we do not observe increases in MAP of more than a few millimeters of mercury in response to injections into the RVLM of ANG II (15) or other angiotensin peptides. Although Fontes and co-workers (6, 7, 28) and Muratini, Averill, and co-workers (4, 21, 22) consistently obtain increases in MAP with unilateral injection of 10–200 pmol of ANG II or ANG(1–7), we have not seen this. The reason for this difference in results is unclear at present but may reflect the details of the different experimental paradigms (e.g., type of anesthesia, ventilation, strain of rat, and specifics of the
microinjection protocol). Nonetheless, the lack of response to angiotensin peptides injected into the RVLM affords us the opportunity to study effects mediated by the sarthran-sensitive receptor without needing to control for independent increases in MAP caused by an action of ANG II and related peptides on the AT$_1$ receptor. This issue may have confused previous studies in that the response to sarthran is not due to blockade of AT$_1$ receptors, whereas the pressor response elicited by ANG II can be blocked totally by AT$_1$ antagonists. This difference in the pharmacology of the depressor response elicited by sarthran and the pressor response reported by others in response to angiotensin peptides suggests that they are distinctly different responses. Thus we have focused on the ability of angiotensin peptides to specifically prevent the effects of sarthran.

Previous studies have used pretreatment with amastatin, an inhibitor of aminopeptidases A and M (1), to distinguish between effects of exogenous ANG II that are mediated directly by ANG II or instead indirectly via its metabolism to fragments of ANG II such as ANG(2–8) or ANG(3–8) (1, 12, 19, 30). Our observation that prior injection of amastatin into the RVLM markedly attenuated the ability of ANG II to block the effects of sarthran suggests that a fragment of ANG II rather than ANG II itself is acting on the sarthran-sensitive receptor. Consistent with this finding, ANG(3–8), a fragment of ANG II that has a very low affinity for the AT$_1$ receptor, was able to completely prevent the sarthran-evoked decrease in MAP. Furthermore, this action of ANG(3–8) was not influenced by prior injection of amastatin. In contrast, Sasaki et al. (27) have previously reported that amastatin injected into the rabbit RVLM did not influence the increase in MAP caused by ANG II. However, as discussed above, the pressor response elicited by injection of ANG II into the RVLM appears to be mediated by AT$_1$ receptors, whereas the effect of sarthran is not. Thus the report by Sasaki et al. (27) that amastatin did not influence the pressor response to injection of exogenous ANG II into the RVLM in rabbits should not be considered to be in conflict with our observations. Rather, it appears that ANG II does not act potently on the sarthran-sensitive receptor responsible for the large decrease in MAP after injection of sarthran into the RVLM.

Because ANG(3–8) injected into the RVLM was able to reverse the effects of sarthran and ANG(3–8) acts preferentially on the AT$_4$ receptor (33), we tested other angiotensin analogs that bind selectively to the AT$_4$ receptor, Nle-ANG IV and divalinal-ANG IV (10, 11, 16). Each of these compounds was able to counteract the depressor response evoked by injection of sarthran into the RVLM. However, several aspects of these responses appear to differ from other AT$_4$ receptor-mediated responses. First, sarthran does not appear to bind to AT$_4$ receptors, at least in a bovine kidney epithelial cell line (10). Though des-[sar$^1$]-sarthran may bind to AT$_4$ receptors (11), such a fragment would not be expected to be produced rapidly in vivo. Second, ANG II is $\sim$100-fold less potent at the AT$_4$ receptor than is ANG IV (33), but ANG II appears to be more potent at reversing the effects of sarthran in the RVLM despite having to be metabolized to be active. Third, divalinal-ANG IV is an antagonist of AT$_4$ receptors in most systems studied (11, 16), whereas in the present studies it acted like an agonist to prevent the sarthran-evoked decrease in AP; however, divalinal-ANG IV has been reported to act like an AT$_4$ agonist in a bovine kidney epithelial cell model (10). Furthermore, ANG(1–7) is the most potent peptide that we have studied to date at preventing the effects of sarthran, and ANG(1–7) does not act on the AT$_4$ receptor (9). Nevertheless, at least in the kidney, ANG(1–7) is readily metabolized to ANG(2–7) and ANG(3–7), both of which bind to the AT$_4$ receptor with an affinity similar to that of ANG IV (9). However, it should be noted that the AT$_4$ receptors most thoroughly studied, those in the rat kidney and bovine adrenal, are likely to have properties that are distinct from AT$_4$ receptors in the brain (34).

The ability of ANG(1–7) to reverse the effects of sarthran was tested on the basis of the reports by Fontes, Santos, and co-workers (6, 7, 25, 28), indicating that ANG(1–7) injected into the RVLM results in an increase in AP distinct from that produced by ANG II. Although in ANG(1–7) injected alone into the RVLM had minimal effects on AP in our studies, it potently attenuated the sarthran-evoked decrease in AP. Interestingly, Fontes et al. (7) noted that unilateral injection into the RVLM of [7-D-Ala]ANG(1–7), a putative antagonist of that ANG(1–7) receptor (25), produced a decrease in MAP of $\sim$10–15 mmHg, which was similar to the response they observed to injection of sarthran. Their data suggest that sarthran may produce some of its effect via the same receptor that binds [7-D-Ala]ANG(1–7), a putative antagonist of that ANG(1–7) receptor (25). However, in the present study, [7-D-Ala]ANG(1–7) did not decrease MAP, in agreement with another study (24).

The present study also addresses two other aspects of the specificity of the response to sarthran. First, not all of the peptide fragments of angiotensin reversed the effects of sarthran. Specifically, ANG(3–7) was ineffective. Second, not all Sar$^1$-containing peptides produced the same effect as sarthran and sarile. We tested Sar-Arg-Gly-Asp-Pro and found that it had no effect on MAP when injected into the RVLM. Furthermore, [Sar$^1$]-ANG II did not produce the same response as sarthran.

In conclusion, sarthran injected into the RVLM of $\alpha$-chloralose-anesthetized rats produces a marked decrease in MAP, and this effect of sarthran is independent of its ability to block AT$_1$ receptors. The site, or sites, within the RVLM at which sarthran acts to evoke the large decrease in MAP has a pharmacological profile distinctly different from other characterized angiotensin receptors. Furthermore, the endogenous ligand at this receptor appears to be something other than ANG II.
REFERENCES


