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Influence of α-chymotrypsin and trypsin on the non-adrenergic non-cholinergic relaxation in the rat gastric fundus

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1 Relaxations of the rat gastric fundus were induced by electrical stimulation of the non-adrenergic non-cholinergic (NANC) neurones, by vasoactive intestinal polypeptide (VIP), by noradrenaline and by isopropylnoradrenaline. The influence of α-chymotrypsin and trypsin thereupon was studied.

2 α-Chymotrypsin 2 u ml^{-1}, present for 30 min, antagonized completely the VIP-induced relaxation, but not the stimulation-induced relaxation; α-chymotrypsin 10 u ml^{-1} also partially antagonized the stimulation-induced relaxation. When α-chymotrypsin 2 u ml^{-1} was added after the relaxation had occurred, it antagonized completely the VIP-induced relaxation, but it also partially antagonized the stimulation-induced relaxation. The partial antagonism of the stimulation-induced relaxation was more pronounced with α-chymotrypsin 10 u ml^{-1}.

3 Trypsin, 10^{-6} M and 3×10^{-6} M, had effects on VIP- and stimulation-induced relaxations similar to those of α-chymotrypsin.

4 The relaxations induced by noradrenaline and isopropylnoradrenaline were not influenced by α-chymotrypsin or trypsin, respectively.

5 The results suggest that a peptide, possibly VIP, is involved in the NANC relaxation of the rat gastric fundus.

Introduction

We have previously shown that exogenous administration of vasoactive intestinal polypeptide (VIP) mimics the relaxation induced by electrical stimulation of the inhibitory non-adrenergic non-cholinergic (NANC) neurones in the rat gastric fundus *in vitro*; VIP therefore is a possible neurotransmitter of these NANC neurones (Lefebvre, 1986). This hypothesis would be strengthened if an antagonist influenced the relaxation obtained in response to NANC neurone stimulation and to exogenous application of VIP in a similar way. As the first descriptions of a VIP antagonist were published only very recently (Laburthe *et al.*, 1986; Pandol *et al.*, 1986), several authors have used peptidases to investigate whether VIP could be the transmitter released from NANC nerve endings (see e.g. Mackenzie & Burnstock, 1980; Altiere & Diamond, 1985). The aim of the present experiments was to study the influence of α-chymotrypsin and trypsin on the relaxation induced by NANC neurone stimulation and by exogenous VIP in the rat gastric fundus. Preliminary accounts of this work have been published (De Beurme & Lefebvre, 1986; Lefebvre, 1986).

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Methods

Longitudinal muscle strips (20 mm long × 3 mm wide) of the gastric fundus of reserpine-treated rats (either sex, 120–370 g, reserpine 5 mg kg^{-1} intraperitoneally 24 h before killing) were prepared as described by Vane (1957); rats were fasted after reserpine administration. The strips were suspended in 18 ml organ baths (load 1 g) containing Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, $CaCl_2$ 1.9, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 10.1) at 37°C, bubbled with 95% O_2 and 5% CO_2. Atropine 10^{-6} M and 5-hydroxytryptamine 3×10^{-6} M were present from the beginning of the experiment.

Strips were equilibrated for 60 min (rinsing every 15 min) and changes in length were recorded auxotonically (Harvard heart-smooth muscle transducer) on a Beckman Type R Dynograph recorder. Transmural stimulation was performed via 2 parallel platinum electrodes (48 mm long, 6 mm wide, 5 mm distance between the electrodes) using a S88 Grass stimulator and a constant voltage unit. Under the experimental conditions used, stimulation at supramaximal voltage, 1 ms duration and 5 Hz frequency induced a maximal relaxation. After the equilibration period, the strips were rinsed every 5–10 min in between drug administration and/or periods of transmural stimulation. The effect of the peptidases was studied on relaxations of similar amplitude induced by NANC neurone stimulation and VIP 10^{-8} M. To exclude non-specific effects of the peptidases, their influence was also studied on relaxation, induced by an agent, not related to peptides or NANC neurones. We have chosen noradrenaline 10^{-6} M–10^{-5} M (in the experiments with α-chymotrypsin) or isopropylnoradrenaline 10^{-8}–10^{-7} M (in the experiments with trypsin); both drugs induce relaxation of the rat gastric fundus via postsynaptic adrenoceptors. The peptidases were administered either 30 min before application of the relaxant stimuli, or 5 min after their application, when the relaxation induced was stable. On each experimental day, 4 strips (from 4 different rats) were used in parallel, 3 receiving and 1 not receiving (control) the peptidase.

Incubation with the peptidases during 30 min

To study the influence of α-chymotrypsin 2 u ml^{-1} or 10 u ml^{-1}, the following protocol was used: with intervals of about 30 min, the relaxation in response to transmural stimulation (applied for approximately 5 min), VIP 10^{-8} M (contact time approximately 5 min) and noradrenaline 10^{-6} to 10^{-5} M (contact time approximately 5 min) was studied. α-Chymotrypsin 2 u ml^{-1} or 10 u ml^{-1} was then added for 30 min (with rinsing and readding the peptidase every 10th min); VIP was then administered, followed after 2 min by transmural stimulation for about 5 min. The strips were then rinsed every 5 min until the original tone was restored. α-Chymotrypsin was then added again and the same sequence was repeated except that, instead of transmural stimulation, noradrenaline was given and left in contact with the tissue for about 5 min.

The same protocol was applied to study the influence of trypsin 10^{-6} M and 3×10^{-6} M except that isopropylnoradrenaline 10^{-8} to 10^{-7} M was given instead of noradrenaline. Relaxant responses were measured at the end of the stimulation time or of the contact time. The relaxation in response to stimulation or to the agonist in the presence of α-chymotrypsin or trypsin was expressed as a percentage of the response obtained in the absence of the peptidase. The relaxation induced by VIP and noradrenaline or isopropylnoradrenaline in the absence of the peptidase was expressed as a percentage of the relaxation in response to transmural stimulation under the same conditions.

Addition of the peptidases after application of the relaxant stimulus

With intervals of about 30 min, the relaxation in response to transmural stimulation (applied for approximately 5 min), VIP 10^{-8} M and either noradrenaline 10^{-6} to 10^{-5} M or isopropylnoradrenaline 10^{-8} to 10^{-7} M (contact time approximately 5 min with each drug) was studied. The relaxant response to VIP, transmural stimulation and either noradrenaline or isopropylnoradrenaline was then studied a second time but the stimulation time and the contact time for VIP, and noradrenaline or isopropylnoradrenaline was increased to about 10 min; about 5 min after starting the stimulation or addition of one of the relaxant drugs, α-chymotrypsin (2 u ml^{-1} or 10 u ml^{-1}) or trypsin (10^{-6} M or 3×10^{-6} M) was added and left in contact with the tissue for 5 min. After washing out the peptidase, the strips were rinsed every 5 min until the original tone was restored.

The relaxant response to a stimulus 5 min after addition of α-chymotrypsin or trypsin, was expressed as a percentage of that just before addition of the peptidase. As for the experiments with 30 min incubation, the relaxation induced by VIP and noradrenaline or isopropylnoradrenaline at the beginning of the experiments was expressed as a percentage of the relaxant response to transmural stimulation.

Drugs

Atropine sulphate (Merck, Brussels, Belgium), α-chymotrypsin (Sigma, St Louis, U.S.A.), (±)-isopropylnoradrenaline hydrochloride (Winthrop, Brussels, Belgium), (−)-noradrenaline bitartrate (Sigma, St Louis, U.S.A.), reserpine (Aldrich Chemie, Brussels, Belgium), 5-hydroxytryptamine creatinine sulphate (Calbiochem-Behring, La Jolla, U.S.A.), trypsin (Sigma, St Louis, U.S.A.), vasoactive intestinal polypeptide (VIP, UCB, Brussels, Belgium) were used. For isopropylnoradrenaline, commercially available ampoules were used. For reserpine, a stock solution was prepared from powder (5 mg ml^{-1} dissolved in 10% ascorbic acid). For trypsin and α-chymotrypsin, a solution was prepared from powder dissolved in distilled water. For VIP, a stock solution was prepared by dissolving 0.5 mg of VIP in distilled water; further dilutions were made the day of the experiment. On the day of the experiment, drugs were kept on ice, and added to the bath in a maximum volume of 0.1 ml.

Statistical analysis

Values are expressed as means with their standard error (s.e.mean). The results obtained in the presence of the two concentrations of each peptidase were compared by means of the two-sample rank test.

Results

The relaxation induced by the concentrations of noradrenaline and isopropylnoradrenaline chosen and by VIP 10^{-8} M at the beginning of the experiment was in general larger (20 to 60%) than that induced by transmural stimulation. Only in 2 series of experiments was the VIP-induced relaxation smaller than the electrically induced one. Expressed as a percentage of the relaxant response to transmural stimulation at the beginning of the experiments, the VIP-induced relaxation ranged from 108.5% to 132.5% in the experiments where α-chymotrypsin was added later and from 93.0% to 126.4% in the experiments where trypsin was added later. Expressed in the same way, the noradrenaline-induced relaxation ranged from 111.8% to 151.2% and the isopropylnoradrenaline-induced relaxation from 121.4% to 164.1%. Relaxations were well maintained during the 5 min periods of stimulation or of contact.

In the control strips, similar results were obtained.

Incubation with the peptidases during 30 min

The addition of the peptidase to the bathing medium increased the tone of the strips. During the 30 min incubation period, the increase in tone slowly disappeared but not always completely. This effect was more pronounced for trypsin than for α-chymotrypsin, and, for both peptidases, for the higher concentration used. The influence of the peptidases on the relaxant stimuli is shown in Table 1. α-Chymotrypsin 2 u ml^{-1} completely prevented the relaxant effect of VIP, but did not influence the relaxant effect of transmural stimulation. α-Chymotrypsin 10 u ml^{-1} completely prevented the relaxant effect of VIP, but it also partially antagonized the relaxation induced by transmural stimulation. When applying transmural stimulation in the presence of α-chymotrypsin, initially a relaxation was induced of similar amplitude to that obtained in the absence of α-chymotrypsin; however, during the 5 min of stimulation, the tone recovered in contrast to the response in the absence of α-chymotrypsin, which remained stable. In the presence of α-chymotrypsin, the relaxant response to noradrenaline was higher than that before.

Similar results were obtained with trypsin. The lower concentration, 10^{-6} M, completely prevented the relaxant response to VIP, but had no influence on the relaxation induced by transmural stimulation. Trypsin 3×10^{-6} M also partially antagonized the effect of

Table 1 Relaxation induced by vasoactive intestinal peptide (VIP), transmural stimulation (TS) and noradrenaline (NA) or isopropylnoradrenaline (Iso) after incubation with α-chymotrypsin or trypsin for 30 min

	VIP	*TS*	*VIP*	*NA*
α-Chymotrypsin				
2 u ml^{-1} ($n = 6$)	0 ± 0	101.8 ± 20.1	0 ± 0	134.1 ± 17.4
10 u ml^{-1} ($n = 6$)	0 ± 0	33.7 ± 16.7*	0 ± 0	151.1 ± 31.5†
	VIP	*TS*	*VIP*	*Iso*
Trypsin				
10^{-6} M ($n = 6$)	0 ± 0	94.7 ± 12.6	0 ± 0	70.4 ± 6.2
3×10^{-6} M ($n = 6$)	2.2 ± 1.8	30.9 ± 10.4*	0.7 ± 0.7	86.0 ± 10.4‡

The relaxant responses are given as a percentage of the response obtained in the absence of the peptidase; the response to VIP is given twice because VIP was administered twice in the presence of the peptidases, once before transmural stimulation and the second time before addition of NA or Iso.

† $n = 5$ and ‡ $n = 4$ for technical reasons.

*Significantly different ($P < 0.05$) from the results in the presence of the lower concentration of the peptidase.

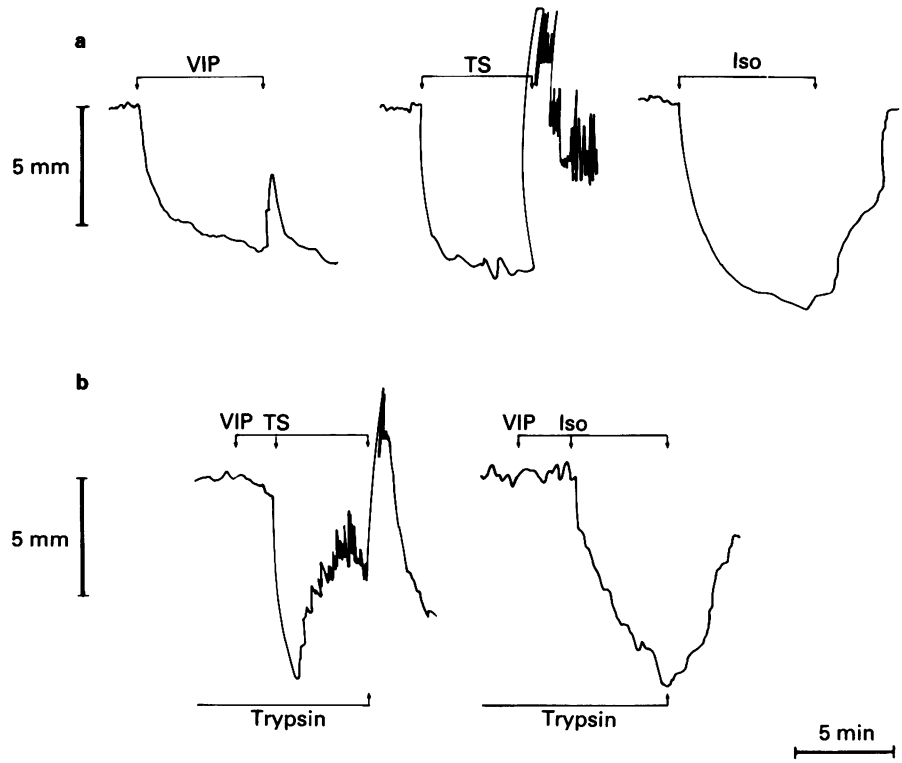

Figure 1 Longitudinal fundus strip of the rat. (a) Relaxation in response to vasoactive intestinal peptide (VIP, 10^{-8} M), to transmural stimulation (TS, supramaximal voltage, 1 ms, 5 Hz) and to isopropylnoradrenaline (Iso, 10^{-7} M). (b) Responses to the same stimuli, 30 min after trypsin 3×10^{-6} M was added to the bathing medium. Transmural stimulation was then applied and isopropylnoradrenaline administered 2 min after administration of VIP.

transmural stimulation (Figure 1). As observed for α-chymotrypsin, transmural stimulation in the presence of trypsin 3×10^{-6} M initially induced a relaxation of similar amplitude to that seen in the absence of the peptidase, but tone recovered during the 5 min of stimulation. In the presence of trypsin, the relaxation induced by isopropylnoradrenaline was lower than that obtained beforehand.

In control strips, the relaxant responses to the different stimuli were reproducible or a tendency to an increase of the responses on repeated application of the stimuli was observed.

Addition of the peptidases after application of the relaxant stimulus

The results of these experiments are given in Table 2. α-Chymotrypsin 2 u ml^{-1} induced an almost complete recovery of the VIP-induced relaxation, but also a partial recovery of the stimulation-induced relaxation. Increasing the α-chymotrypsin concentration to 10 u ml^{-1} induced a larger but incomplete recovery of the electrically evoked relaxation (Figure 2). Relaxation by noradrenaline was not influenced by α-chymotrypsin.

The lower concentration of trypsin (10^{-6} M) induced a complete recovery of the VIP-induced relaxation, but also a pronounced recovery of the stimulation-induced relaxation. Administration of the higher trypsin concentration, 3×10^{-6} M, induced a complete recovery of the VIP-induced relaxation; the recovery of the stimulation-induced relaxation by adding trypsin 3×10^{-6} M was still more pronounced than that seen with the lower concentration but the difference was not significant. The isopropylnoradrenaline-induced relaxations were not influenced by trypsin. In control strips, the relaxant responses to the different stimuli were reproducible.

Table 2 Relaxation induced by vasoactive intestinal peptide (VIP), transmural stimulation (TS) and noradrenaline (NA) or isopropylnoradrenaline (Iso) 5 min after addition of α-chymotrypsin or trypsin

	VIP	*TS*	*NA*
α-Chymotrypsin			
2 u ml^{-1} ($n = 6$)	5.9 ± 3.8	82.8 ± 5.4	96.9 ± 2.3
10 u ml^{-1} ($n = 7$)	0.4 ± 0.4	33.4 ± 13.8**	99.6 ± 5.9
	VIP	*TS*	*Iso*
Trypsin			
10^{-6} M ($n = 7$)	0 ± 0	38.4 ± 10.2	99.7 ± 3.5
3×10^{-6} M ($n = 6$)	0 ± 0	9.4 ± 5.1	100.7 ± 5.0

α-Chymotrypsin or trypsin was added when the relaxation induced by a stimulus was stable (5 min after its application); 5 min after the addition of α-chymotrypsin or trypsin, the relaxation was measured and expressed as a percentage of that measured just before the addition of the peptidase.
**Significantly different ($P < 0.01$) from the results in the presence of the lower concentration of the peptidase.

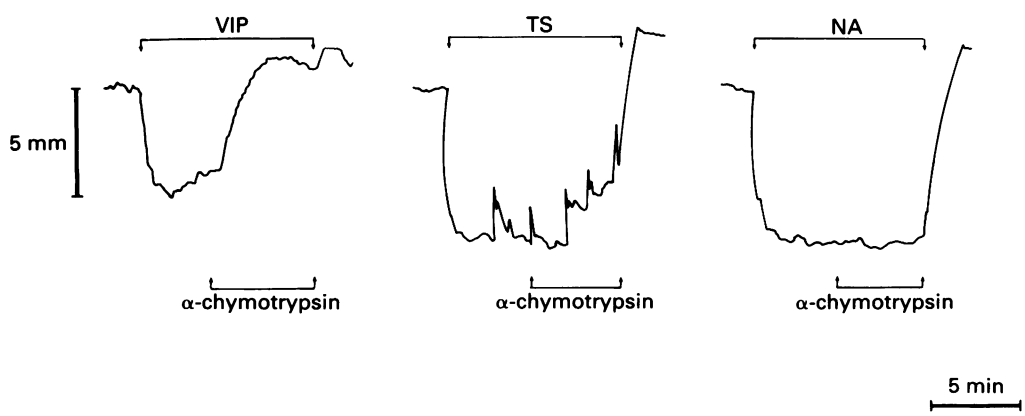

Figure 2 Longitudinal fundus strip of the rat. Relaxations were induced by vasoactive intestinal peptide (VIP, 10^{-8} M), transmural stimulation (TS, supramaximal voltage, 1 ms, 5 Hz) and noradrenaline (NA, 10^{-6} M). Approximately 5 min after application of a relaxant stimulus, α-chymotrypsin 10 uml^{-1} was added and left in the bathing medium for 5 min.

Discussion

The relaxation in response to NANC neurone stimulation in the rat gastric fundus is mimicked by VIP (Lefebvre, 1986). To investigate further the possibility that VIP is the NANC neurotransmitter involved in this response, the influence of the peptidases α-chymotrypsin and trypsin was studied on the relaxation induced by NANC neurone stimulation and by exogenous VIP. α-Chymotrypsin and trypsin cleave peptides respectively at the level of tyrosine and of lysine and arginine; these 3 amino acids are present in the VIP sequence. To exclude non-specific effects of the peptidases, their influence was also studied on the relaxation induced by noradrenaline and isopropylnoradrenaline: these drugs induce relaxation by interaction with the postsynaptic α- and β-(noradrenaline) and β-adrenoceptors (isopropylnoradrenaline), which have been described in the rat gastric fundus (Lefebvre *et al.*, 1984; Verplanken *et al.*, 1984).

The influence of α-chymotrypsin (administered beforehand) on VIP- and NANC neurone-induced relaxations has already been studied in different preparations: e.g. guinea-pig taenia coli (Mackenzie & Burnstock, 1980), canine gastric muscularis mucosae (Angel *et al.*, 1983), pig bladder neck (Hills *et al.*, 1984), cat airways (Altiere & Diamond, 1985), rat duodenum (Manzini *et al.*, 1985) and ileum (Manzini *et al.*, 1986). Only in the canine gastric muscularis mucosae, superfusion with α-chymotrypsin (10 u ml^{-1}) clearly antagonized the inhibitory effect of NANC nerve stimulation. In the other preparations, α-chymotrypsin (1 or 2 u ml^{-1}) antagonized the relaxations induced by exogenous VIP but not those by NANC nerve stimulation. Several explanations seem possible: (1) VIP is not involved in the response to NANC nerve stimulation; (2) a peptide not serving as a substrate for α-chymotrypsin is involved; (3) VIP is involved but escapes degradation by α-chymotrypsin because this enzyme reaches the synaptic cleft with difficulty due to its large size.

The latter possibility might explain why higher concentrations of a peptidase are needed to antagonize endogenously released neurotransmitter than to antagonize exogenous VIP. In our experiments, α-chymotrypsin 2 u ml^{-1}, incubated for 30 min, antagonized completely the VIP-induced relaxation but not the stimulation-induced relaxation, while 10 u ml^{-1} α-chymotrypsin also markedly antagonized the latter relaxation. This antagonism of the stimulation-induced relaxation seems not to be a non-specific effect of the peptidase as the noradrenaline-induced relaxation was not antagonized. The relaxation in response to noradrenaline was actually increased in the presence of α-chymotrypsin, but this is probably due to some spontaneous change in response to the same stimulus. We have no explanation for the observation that, notwithstanding the incubation of the peptidase for 30 min, transmural stimulation induces at first a relaxation of similar amplitude as that obtained in the absence of the peptidase. Even after 5 min of stimulation, degradation of endogenously released neurotransmitter seems not to be complete, as a certain degree of relaxation persisted.

We also investigated the influence of α-chymotrypsin administered once the relaxation in response to a stimulus had been established. In contrast to what we expected, α-chymotrypsin, administered acutely, antagonized the stimulation-induced relaxation to the same extent as when already present for 30 min. Even the lower α-chymotrypsin concentration had a small antagonistic effect on the stimulation-induced relaxation, without influencing the relaxations evoked by noradrenaline. These results thus showed that the proteolytic enzyme α-chymotrypsin is able to antagonize partially the NANC relaxation in the rat gastric fundus; this suggests that a peptide, possibly VIP, could be involved. The observation that transmural stimulation after 30 min incubation with the peptidase initially induced a relaxation of similar amplitude to that seen in the absence of the peptidase, might suggest that a non-VIP component is also involved in the NANC relaxation in this preparation.

The results with trypsin were very similar to those obtained with α-chymotrypsin; the effect of trypsin on the stimulation-induced relaxation was clearly more pronounced when the peptidase was administered after the relaxation had been established. As the same tendency was seen in the experiments with α-chymotrypsin, this observation suggests that adding a peptidase during an established relaxation is a better way to study its influence on endogenously released neurotransmitter. The small influence of trypsin when incubated for 30 min on the isopropylnoradrenaline-induced relaxations, is probably due to some decline in the isopropylnoradrenaline-induced responses in these experiments. Indeed, the effect was less pronounced for the higher concentration of trypsin and was not seen when trypsin was added during an isopropylnoradrenaline-induced relaxation.

Both peptidases induced an increase in tone of non-relaxed strips. Contractile responses to similar concentrations of α-chymotrypsin and trypsin as used in our experiments have already been described in the rat gastric fundus strip preparation; tachyphylaxis developed rapidly (Gilfoil & Kelly, 1966). This contractile effect of the proteolytic enzymes cannot explain their influence on VIP- and stimulation-induced relaxations, when administered once the relaxation had been established, because the same effect should then have been seen when administering the peptidases during a noradrenaline- or isopropylnoradrenaline-induced relaxation.

Our results thus provide further evidence that VIP is

involved in the NANC relaxation in the rat gastric fundus. As neither trypsin nor α-chymotrypsin is specific in degrading VIP, we cannot exclude the possibility that another peptide is released during NANC neurone stimulation in the rat gastric fundus. However, of the neuropeptides present in the rat stomach wall which have been suggested as being involved in inhibitory NANC relaxant responses in other preparations, only VIP mimicked the response to NANC neurone stimulation in the rat gastric fundus (Lefebvre, 1986).

We conclude that the inhibitory influence of the proteolytic enzymes α-chymotrypsin and trypsin on the NANC relaxation in the rat gastric fundus provides evidence that a peptide, possibly VIP, is involved.

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