Tachykinin receptors mediating responses to sensory nerve stimulation and exogenous tachykinins and analogues in the rabbit isolated iris sphincter

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1 We have used selective tachykinin receptor agonists and antagonists to investigate the nature of the receptors mediating responses to endogenous and exogenous tachykinins in the rabbit iris sphincter preparation *in vitro*.

2 The NK₁-selective agonist, substance P methyl ester, induced contraction with a pD₂ of 9.16 indicating the presence of NK₁ receptors. In confirmation, the NK₁-selective antagonist, GR82334, competitively antagonized responses to substance P methyl ester with high affinity (pK_B 7.46).

3 NK₃ receptors also mediate contraction since NK₃-selective agonists exhibited high potency, e.g. the pD₂ of [Me-Phe⁷]-neurokinin B was 9.67, and their responses were not inhibited by GR82334 (10 µM).

4 NK₂ receptor activation does not seem to contribute to contraction since the NK₂-selective agonist [β-Ala⁶]-neurokinin A(4-10) had relatively low potency (pD₂ 6.43), and the NK₂-selective antagonists MEN10207 (1 µM) and L-659,877 (10 µM) were inactive or had low affinity, respectively.

5 GR82334 (1 µM) significantly inhibited responses to electrical field-stimulation of non-adrenergic non-cholinergic sensory nerves (3, 10 and 30 Hz), and caused a rightward shift of the log concentration-response curve to bradykinin (lateral shift *ca.* 1000 fold). Higher concentrations of GR82334 (10 µM) significantly attenuated responses to capsaicin (1–60 µM) whilst completely abolishing responses to field-stimulation (3, 10 and 30 Hz) and bradykinin (1 nM–3 µM).

6 In conclusion, NK₁ and NK₃ receptor activation results in contraction of the rabbit iris sphincter. The contractile response following sensory nerve stimulation by bradykinin, capsaicin and electrical field stimulation results from NK₁ receptor activation.

Keywords: Tachykinin receptors; neurokinin; iris sphincter; bradykinin; capsaicin; substance P; sensory nerves; NK₁ antagonist; GR82334; NANC responses.

Introduction

Sensory, non-adrenergic non-cholinergic (NANC), nerve stimulation of the rabbit iris sphincter *in vitro* by electrical field-stimulation, capsaicin or bradykinin, results in contraction of the smooth muscle. Functional studies suggest these responses are caused by nerve-released tachykinins (Butler & Hammond, 1980; Muramatsu *et al.*, 1987), although the identity of the tachykinins released by each of the sensory nerve stimulants remains unclear. Our previous work with selective tachykinin receptor agonists (Hall *et al.*, 1991) and antagonists (Hall *et al.*, 1992b) indicated that activation of NK₁ and NK₃ postjunctional receptors results in contraction in this preparation; while radioligand binding studies have demonstrated specific binding sites for substance P in iris sphincter muscle membrane preparations (Too *et al.*, 1988; Denis *et al.*, 1991). In principle, therefore, an interaction of neuronally-released tachykinins with either or both NK₁ and NK₃ receptor types could contribute to contractile responses following sensory nerve stimulation.

The aim of the present study was two fold. First, to confirm the nature of the tachykinin receptors in this preparation, by use of recently available agonists and antagonists that are selective for the individual tachykinin receptors. Second, to investigate the effect of NK₁-selective antagonists on responses to NANC electrical field-stimulation, and to chemo-stimulation by capsaicin and bradykinin. Preliminary accounts of this work have been published (Hall *et al.*, 1992b; 1993).

Methods

Tissue preparation and recording arrangements

Male New Zealand albino rabbits (2.5–3.0 kg) were killed by an overdose of i.v. pentobarbitone sodium (Sagatal). The eyes were enucleated immediately after death and opened by an incision 2–3 mm dorsal to the limbus, followed by excision of the iris from the ciliary margin. The sphincter pupillae muscles were dissected free of dilator muscle and mounted intact, either on stainless-steel tissue-holders or between parallel platinum electrodes, in 2 ml silanised glass organ baths in Krebs solution at 37°C. The preparations were attached to isometric Grass FT03B force-displacement transducers under an initial resting tension of 150 mg. Mechanical activity was recorded on Grass model 7E polygraphs. Electrical field stimulation was applied by Scientific Research Instruments Limited (SRI 6051) stimulators.

The composition of the Krebs solution was (mM): Na⁺ 140, K⁺ 5.9, Cl⁻ 104.8, H₂PO₄⁻ 1.2, HCO₃⁻ 24.9, Ca²⁺ 2.6, Mg²⁺ 1.15, SO₄²⁻ 1.15, glucose 10. The Krebs solution was maintained at pH 7.4 by constant bubbling with 95% O₂: 5% CO₂. In all experiments the Krebs solution contained hexamethonium (100 µM), mepyramine, cimetidine, guanethidine, atropine and ibuprofen (all 1 µM).

Experimental protocols

An equilibration period of 60 min was allowed before commencing regular dosing, after which time the maximal response to carbachol was established. Concentration-response curves for bradykinin were determined at the start, and for substance P at the end, of experiments and a concentration-response curve to carbachol was obtained in each prepara-

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tion. Cumulative concentration-response curves were established to stimulants with 20 min allowed between agonists, and when several different tachykinins and analogues were used they were applied in a randomized order between preparations. Antagonist incubation time was 5 min, since increasing this to 15 min had no effect on affinity estimates for GR82334 and MEN10207. Electrical field-stimulation was carried out with square-wave pulses of 0.3 ms duration at 50 V for 15 s with 15–20 min between each stimulation period.

Effect of tachykinin receptor agonists and antagonists

The receptor selectivity of the tachykinin analogues used in this study is as follows: substance P methyl ester and GR73632 (NK₁ agonists); [β -Ala⁸]-NKA(4-10) and GR64349 (NK₂ agonists); senktide and [Me-Phe⁷]-NKB (NK₃ agonists), GR82334 (NK₁ antagonist), MEN10207 and L-659,877 (NK₂ antagonists). In all experiments, paired irises from the same animal were used. Full concentration-response curves were obtained for bradykinin, mammalian tachykinins and analogues in each series either in the absence (concurrent control), or the presence (test) of the antagonist (1, 3.2 or 10 μ M). For substance P methyl ester control concentration-response curves were obtained followed by those obtained in the presence of increasing concentrations of GR82334 (1, 3.2 and 10 μ M; test tissues), or no antagonist (concurrent time-control tissues). Full concentration-response curves for carbachol and [Arg⁸]-vasopressin were obtained in the absence and presence of GR82334 (30 μ M).

Effect of GR82334 on responses to electrical field-stimulation and capsaicin

One paired preparation acted as a concurrent control, with the other as a test preparation. Responses to carbachol, a submaximal concentration of substance P methyl ester (10 nM), and electrical field-stimulation (3, 10 and 30 Hz) were obtained, and these were then repeated in the presence of 1 μ M GR82334, and then 10 μ M GR82334. Finally, a concentration-response curve was established for capsaicin in the presence of GR82334 (10 μ M). The concurrent control preparation received an identical dosing protocol, but in the absence of GR82334 throughout. In a further series of experiments, the effect of a higher concentration of GR82334 (30 μ M) was tested against responses to capsaicin only.

Source of agents used

Agents were obtained as follows: Sagatal (RMB Animal Health Ltd, U.K.), carbachol, capsaicin, hexamethonium bromide, ibuprofen, phosphoramidon (Sigma, U.K.), mepyramine maleate (May & Baker, U.K.), guanethidine sulphate (CIBA, U.K.), substance P, substance P methyl ester, senktide (Succ-[Asp⁶,Me-Phe⁸]-substance P(6-11)), neurokinin A, neurokinin B, [Arg⁸]-vasopressin, and bradykinin (Peninsula Laboratories, Europe; or Bachem, U.K.), [Me-Phe⁷]-neurokinin B ([Me-Phe⁷]-NKB; Nova Biochem, U.K.), cyclo(Gln,Trp,Phe,Gly,Leu,Met) (L-659,877, Cambridge Research Biochemicals, U.K.), δ -amino valeryl-[L-Pro⁹,N-MeLeu¹⁰]-substance P(7-11) (GR73632), [Lys³,Gly⁸-R- γ -Lactam,Leu⁹]-neurokinin A(3-10) (GR64349) and [D-Pro⁹ [Spiro- γ -Lactam] Leu¹⁰,Trp¹¹]-physalaemin(1-11) (GR82334) (Peninsula Laboratories, Europe; or Glaxo Group Research, U.K.), [β -Ala⁸]-neurokinin A(4-10); [β -Ala⁸]-NKA(4-10), A. Menarini Pharmaceuticals, Italy; or Bachem, U.K.), [Tyr⁵,D-Trp^{6,8,9},Arg¹⁰]-neurokinin A(4-10) (MEN10207) (A. Menarini Pharmaceuticals, Italy; or Peninsula Laboratories, Europe). All salts were of analytical grade and were obtained from B.D.H., U.K.

L-659,877, capsaicin, neurokinin B, [Me-Phe⁷]-NKB, senktide and [β -Ala⁸]-NKA(4-10) were dissolved in dimethylsulphoxide to 1 mM stock. Ibuprofen was dissolved in 5%

Na₂CO₃. All other agents, and all dilutions, were made up in distilled water and peptides were stored at -20°C. Dimethylsulphoxide (maximum amount injected during experiments) did not affect basal tension *per se*, nor concentration-response curves to neurokinin A ($n = 4$, data not shown).

Expression of results and statistical analysis

For agonists, responses are expressed in terms of % maximal response to carbachol in individual preparations, determined at the start of experiments. Data for individual experiments were combined and analysed in two ways. To allow conventional graphical display, responses were calculated for each concentration of test agonist in individual experiments (control tissues from all the antagonist experiments described below) and plotted as mean responses $\pm$ s.e.mean. For the purposes of obtaining objective slope and EC₅₀ estimates, individual agonist concentration-response relationships were analysed by determining the least-squares fit of data to the logistic function $y = y_{\max} A^p / (A^p + A_{50}^p)$, where y is the response (expressed in terms of maximum responses to carbachol), $y_{\max}$ is the estimated maximum response to that tachykinin, A is the tachykinin concentration, A_{50} is the concentration producing 50% of that agonist's maximum response (the EC₅₀), and p is a slope factor (equivalent to the Hill Slope). These curve-fitting procedures were carried out using Sigma Plot 4.1 computer software (Jandel Scientific, CA, USA).

For antagonists, the pK_B estimates were determined from individual dose-ratios using the Gaddum-Schild equation, $-\log K_B = pK_B = \log(x-1) - \log[A]$, where x is the dose-ratio and $[A]$ the applied antagonist concentration. Affinities are described as pK_B values when Schild plot analysis showed no significant departure from unity slope, or as apparent pK_B values when such analysis could not be carried out because a limited antagonist concentration-range was used (see Results). Dose-ratios were estimated between the responses in test and concurrent-control tissues, except in experiments to estimate the effect of GR82334 against responses to substance P methyl ester. In all cases n represents data obtained from 4–17 animals and refers to either the number of estimates (pK_B) or the number of preparations (all other estimates). Error estimates are given as s.e.mean. Tests for statistical significance were carried out with paired or unpaired t tests, or analysis of variance, as appropriate.

Results

Agonist potencies

Agonist potencies are shown in Table 1. The NK₁-selective agonist substance P methyl ester and the NK₃-selective agonist [Me-Phe⁷]-NKB had high potency, whereas the NK₂-selective agonist [β -Ala⁸]-NKA(4-10) was *ca.* 1000 fold less active. The mammalian tachykinins substance P, neurokinin A and neurokinin B were of similar potency (pD_2 estimates were 8.45, 8.64 and 8.88 respectively). All tachykinins and analogues reached similar maximal responses, which did not significantly differ from the maximal response obtained for carbachol ($P > 0.05$) (Table 1 and Figure 1). Estimates of the logistic slope factor (p) varied significantly ($P < 0.05$) between tachykinins as a whole; but after exclusion of the apparently non-sigmoidal [β -Ala⁸]-neurokinin A(4-10) curve, there were no other significant differences ($P > 0.05$) in useful parameter comparisons by analysis of variance.

Antagonist studies

The NK₁-selective antagonist GR82334 (1, 3 and 10 μ M), caused a parallel shift of the log concentration-response

Table 1 Activities of tachykinins and analogues in contracting the rabbit iris sphincter preparation

Tachykinin	n	Slope	Maximum (%)	pD_2	EC_{50} [nM]
Substance P	13	0.73 ± 0.10	92.1 ± 4.3	8.45 ± 0.26	3.5
Neurokinin A	9	0.84 ± 0.10	106.4 ± 5.7	8.64 ± 0.20	2.3
Neurokinin B	6	0.75 ± 0.11	106.5 ± 4.2	8.88 ± 0.17	1.3
Substance P methyl ester	17	0.62 ± 0.04	100.4 ± 4.8	9.16 ± 0.10	0.7
[Me-Phe ⁷]-neurokinin B	4	0.43 ± 0.04	87.7 ± 4.7	9.67 ± 0.21	0.2
[β -Ala ⁸]-neurokinin A (4-10)	5	1.12 ± 0.18	97.2 ± 6.0	6.43 ± 0.12	370.7

Experiments were carried out in the presence of hexamethonium (100 μ M), guanethidine, mepyramine, cimetidine, atropine and ibuprofen (all 1 μ M). Logistic parameter estimation procedures are described in Methods. Maxima are expressed in terms of % carbachol maximum, and none differed significantly from 100% ($P > 0.05$). Slopes overall differed significantly between tachykinins ($P < 0.05$).

Figure 1 Log concentration-response curves for tachykinins and analogues in rabbit iris sphincter. In (a) responses to the mammalian tachykinins substance P (●), neurokinin A (■) and neurokinin B (▲) and in (b), responses to the NK₁-selective agonist [Me-Phe⁷]-neurokinin B (●), the NK₁-selective agonist substance P methyl ester (●) and the NK₂-selective agonist [β -Ala⁸]-neurokinin A(4-10) (▲). Responses are shown as % maximal response to carbachol (CCh) and each point shows the mean ($\pm$ s.e.mean) obtained from 4–17 individual preparations. The broken lines represent mean logistic fit log concentration-response curves calculated from the parameters in Table 1. Note some deviations from the logistic model (see text).

curve to the NK₁-selective agonist substance P methyl ester, and the Schild regression did not differ significantly from unity (0.87 ± 0.11 $P > 0.05$), compatible with a competitive antagonism at a single site, for which the pK_B estimate was 7.46 ± 0.14 ($n = 12$). However, when tested against

neurokinin A, GR82334 (10 μ M) showed antagonism (in 4 out of 6 preparations) only of the lower portion of that agonist's log concentration-response curve, from which an apparent pK_B estimate of 6.25 ± 0.23 could be calculated (data not shown). GR82334 (3 μ M) did not antagonize responses to substance P and senktide (i.e. apparent $pK_B < 5.5$), carbachol or [Arg⁸]-vasopressin (data not shown).

The NK₂-selective antagonist MEN10207 (1 μ M) did not inhibit responses to [β -Ala⁸]-NKA(4-10), GR64349 and substance P. The potent NK₂-preferring antagonist, L-659,877, inhibited responses to GR64349 and GR73632 but with low affinity (apparent pK_B values 6.29 ± 0.29 , $n = 5$, and 6.37 ± 0.34 , $n = 7$, respectively), and was inactive (10 μ M, $n = 4$) against responses to substance P.

Effect of GR82334 on responses to sensory nerve stimulation

The NK₁-selective antagonist GR82334 (1 μ M) attenuated responses to sensory NANC field-stimulation, (Figure 2a), bradykinin (Figure 2b), and submaximal responses to substance P methyl ester used as a control (Figure 2c). At 10 μ M, GR82334 attenuated responses to capsaicin (Figure 2d), abolished responses to both bradykinin (Figure 2b) and to substance P methyl ester (Figure 2c), and reversibly abolished responses to electrical field-stimulation (Figure 2a). At a higher concentration (30 μ M), GR82334 abolished responses to capsaicin (Figure 2d).

Discussion

Our findings in this study indicate a role for NK₁ and NK₃, but not NK₂, receptors mediating tachykinin-evoked contractile responses in the rabbit isolated sphincter preparation. Further, our observations with the NK₁ antagonist, GR82334, suggest a role for NK₁ receptors in mediating contractions following tachykinin-release evoked from sensory NANC neurones either by electrical field-stimulation or chemo-stimulation by bradykinin and by capsaicin.

Tachykinin receptor types in the rabbit iris sphincter

Results from this study using agonists selective for the NK₁, NK₂ and NK₃ receptor types support our earlier findings (see Introduction) suggesting that a mixed NK₁ and NK₃ receptor population mediates smooth muscle contraction to exogenous tachykinins and analogues in the isolated iris sphincter preparation of the rabbit. Thus, in agreement with our previous studies (Hall *et al.*, 1991) using the novel NK₁-selective agonist GR73632 (Hagan *et al.*, 1991b), the established NK₁-selective agonist substance P methyl ester (Watson *et al.*, 1983) has relatively high potency (pD_2 9.16). This suggested presence of NK₁ receptors was confirmed with the NK₁-selective antagonist GR82334 (Hagan *et al.*, 1991a). Schild plot analysis of the antagonism by GR82334 of res-

ponses to substance P methyl ester, indicated a competitive interaction with an NK₁-receptor population, yielding a pK_B estimate of 7.46. This value is similar to estimates of the affinity of this antagonist at NK₁ receptors in the guinea-pig ileum (pK_B 7.64, Hagan *et al.*, 1991a; pK_B 7.42, Hall *et al.*, 1992a), guinea-pig trachea (pK_B 7.23; Hagan *et al.*, 1991a) and guinea-pig proximal urethra (pK_B 7.31; Maggi & Patacchini, 1992). Our affinity estimate for GR82334 (pK_B 7.46) is, however, higher than that reported by Beresford *et al.* (1992) in the rabbit iris (pK_B 6.11): the reason for this discrepancy is not known.

Activation of NK₃-receptors also appears to cause contraction of the iris sphincter since the NK₃-selective agonists senktide (Hall *et al.*, 1991) and [Me-Phe⁷]-NKB (Drapeau *et al.*, 1987) (pD_2 9.67) have high potency. However, it should be noted that the evidence for the existence of NK₃-receptors is the high activity of NK₃-selective agonists, together with the absence of antagonism of these responses by GR82334. Confirmation through the use of NK₃-selective receptor antagonists is required.

With respect to NK₂ receptors, the NK₂-selective agonist [β -Ala⁸]-NKA(4-10) (Rovero *et al.*, 1989) had low potency (pD_2 6.43), in agreement with our earlier studies (Hall *et al.*, 1991) with the NK₂-selective agonist GR64349 (Hagan *et al.*, 1991b). In view of the low potencies of NK₂-selective agonists compared to those seen in defined NK₂ receptor systems (Rovero *et al.*, 1989; Hagan *et al.*, 1991b; Hall *et al.*, 1992a), it seems most likely that at the high concentrations necessary to obtain responses, these agonists are interacting with non-NK₂ receptors. This interpretation is supported by our demonstration of the lack of antagonism by the NK₂-selective antagonist, MEN 10207 (Maggi *et al.*, 1990; Rovero *et al.*, 1990), and the low affinity of the NK₂-selective antagonist, L-659,877 (McKnight *et al.*, 1991) when tested against NK₂-selective agonists. The latter antagonist also inhibited responses to the NK₁-selective agonist, GR73632 when used at these high concentrations. In fact, a low affinity of L-659,877 for NK₁ or NK₃ receptors has previously been demonstrated (Williams *et al.*, 1988). We therefore conclude that NK₂ receptors are not present in rabbit iris sphincter.

Effect of GR82334 on responses to mammalian tachykinins and sensory NANC nerve stimulation

Both neurokinin A and substance P are released from peripheral termini of mammalian primary afferent neurones (see Holzer, 1988). In the iris sphincter, the NK₁-selective antagonist, GR82334, inhibited some part of responses to neurokinin A, though not to substance P. The apparent affinity estimate for GR82334 obtained with neurokinin A as the agonist, was much lower than when tested against substance P methyl ester. However, since neurokinin A shows little selectivity between tachykinin receptor types, and is

Figure 2 The effect of GR82334 on responses to sensory nerve stimulation. Mean responses ($\pm$ s.e.mean) are shown for 7 preparations. In (a) responses to sensory non-adrenergic non-cholinergic electrical field-stimulation at 3, 10 and 30 Hz was determined in the absence (solid columns) and presence of GR82334 at 1 μ M (diagonally hatched columns) and 10 μ M (horizontally hatched columns). In (b) log concentration-response curves to bradykinin (●) control; and in the presence of (○) 1 μ M, and (Δ) 10 μ M GR82334. In (c) control submaximal responses to substance P methyl ester (SPOMe) are shown (solid column), and after GR82334 treatment (diagonally hatched column) 1 μ M, and (third column) 10 μ M. In (d) (●) control log concentration-response curves for capsaicin; and in the presence of GR82334 at 10 μ M (○), and 30 μ M (Δ). Experiments were carried out in Krebs solution containing hexamethonium (100 μ M), atropine, mepyramine, cimetidine, guanethidine and ibuprofen (all 1 μ M). Panels (a), (b) and (d) are reproduced from Hall *et al.* (1993) with permission from the Publishers.

active at NK₃ receptors it seems likely that the relative lack of antagonism is due to an interaction of this agonist with NK₃ receptors and that this component of the neurokinin A response is resistant to antagonism by GR82334. This suggestion of an action of neurokinin A at two sites is supported by the finding that in most preparations GR82334 (10 μ M) caused a parallel shift of only the lower portion of the log concentration-response curve. However, with substance P, the lack of any demonstrable antagonism of responses was unexpected because, although this agonist is not particularly selective between the three tachykinin receptors, it is thought to show a preference for the NK₁ type (see Hall *et al.*, 1991; 1993). However, one explanation for this discrepancy comes from preliminary data suggesting that the predominant component of the action of substance P is through an interaction with a further 'atypical' site from which the offset kinetics are very slow (Hall *et al.*, 1993): however, after phenoxybenzamine pretreatment this site is no longer evident, and GR82334 now antagonizes substance P with the affinity that might be expected at NK₁ receptors (Hall *et al.*, 1993).

GR82334 inhibited responses to three different modes of sensory nerve stimulation, namely by bradykinin, sensory NANC electrical field stimulation and by capsaicin. GR82334 (1 μ M) attenuated responses to 3, 10 and 30 Hz electrical field-stimulation and shifted the log concentration-response curve for bradykinin *ca.* 1000 fold to the right. At a higher concentration (10 μ M), GR82334 abolished responses to bradykinin and to electrical field-stimulation. These results suggest that both bradykinin and electrical field-stimulation contract the iris sphincter through the release of tachykinins that act on NK₁ receptors, but argue against a major role of substance P contributing to these responses (see above). GR82334 (10 μ M) inhibited contractile responses to capsaicin,

and at still higher concentrations (30 μ M) abolished responses to this sensory nerve stimulant. The higher concentration of GR82334 required for total inhibition of responses to capsaicin may be a consequence of the different mechanism of excitation by this agent, differential access to transmitter pools and amounts of released peptide, (Håkanson *et al.*, 1987; Wang & Håkanson, 1992a; Hall *et al.*, 1993). Though it is of interest that the potent non-peptide NK₁ antagonist ($\pm$)-CP-96,345 also inhibits responses to capsaicin and electrical field-stimulation in this preparation (Håkanson *et al.*, 1991; Wang & Håkanson, 1992b), it does so at relatively high concentrations (e.g. pIC₅₀ 5.4 for nerve field-stimulation; Wang & Håkanson, 1992c), when the established non-specific actions of the antagonist in this and other preparations (Hall *et al.*, 1992a; Wang & Håkanson, 1992c) complicates interpretation. It is of interest that GR82334 was able to inhibit completely responses to bradykinin, capsaicin, and electrical field-stimulation which suggests that under these conditions only NK₁-receptors are involved in mediating contractile responses. A similar conclusion has been drawn previously (Wang & Håkanson, 1992b).

In conclusion, NK₁ receptors are present in the rabbit isolated iris sphincter preparation, and their activation results in contraction of the smooth muscle. NK₃ receptor stimulation also leads to contraction. The contractile responses seen following sensory nerve-stimulation by electrical field stimulation, bradykinin and capsaicin are mediated via NK₁-receptor activation.

We wish to thank Dr C.A. Maggi, A. Menarini Pharmaceuticals, Italy, for the gifts of [β -Ala⁸]-NKA(4-10) and MEN10207, and Drs R.M. Hagan and S.J. Ireland, Glaxo Group Research, UK, for GR82334, GR73632 and GR64349. This study was supported by the Wellcome Trust.

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(Received January 8, 1993)

Revised February 16, 1993

Accepted March 17, 1993)