Investigations of the dual contractile/relaxant properties showed by antioquine in rat aorta

M. Dolores Ivorra, *Claire Lugnier, Maria Catret, Elsa Anselmi, Diego Cortes & ¹Pilar D'Ocon

Laboratorio de Farmacognosia y Farmacodinamia, Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia, Avda Vicent Andrés Estelles, s/n 46100 Burjasot, Valencia, Spain and *Laboratoire de Pharmacologie Cellulaire et Moléculaire, Faculté de Pharmacie, Université Louis Pasteur CNRS URA 600, BP 24, 67401 Illkirch, France

1 In the present study we assessed the activity of antioquine, a bisbenzyltetrahydroisoquinoline alkaloid isolated from *Pseudoxandra sclerocarpa*, by examining its effects on the contractile activity of rat isolated aorta, specific binding of [³H]-(+)-*cis*-diltiazem, [³H]-nitrendipine and [³H]-prazosin to cerebral cortical membranes and the different molecular forms of cyclic nucleotide phosphodiesterases (PDE) isolated from bovine aorta.

2 Contractions in rat aorta induced by high concentrations of KCl (80 mM) and noradrenaline (1 μ M) were inhibited by antioquine in a concentration-dependent manner (0.1 μ M–300 μ M). The alkaloid appeared more potent against KCl-induced contractions. This inhibitory effect was observed at both 37°C and 25°C.

3 Paradoxically, at the highest concentration tested (300 μ M) antioquine induced a contractile response of similar magnitude in the presence and absence of extracellular calcium, at 37°C. This activity was greatly attenuated at 25°C. Antioquine-induced contractions were not inhibited by prazosin (0.1 μ M), nifedipine (1 μ M) or diltiazem (100 μ M). On the contrary, prazosin and nifedipine slightly increased the contractions in the presence of extracellular calcium. Papaverine (100 μ M) partially inhibited the contractile response to antioquine both in the presence and absence of extracellular calcium.

4 At 25°C, in Ca²⁺-free solution, antioquine (300 μ M) did not modify the contractile response (phasic and tonic) evoked by noradrenaline, but increased the phasic contraction induced by caffeine. At 37°C, the contraction elicited by antioquine made it impossible to observe the noradrenaline-induced one.

5 Antioquine showed affinity for the [³H]-prazosin binding site and for the [³H]-(+)-*cis*-diltiazem binding site of the Ca²⁺-channel receptor complex, but had no effect at the dihydropyridine binding site in rat cerebral cortex.

6 Antioquine weakly inhibited some PDE forms isolated from bovine aorta: a CaM-PDE (PDE I) which preferentially hydrolyzes cyclic GMP and is activated by calmodulin, and a rolipram-sensitive cyclic AMP-PDE (PDE IV) which hydrolyzed cyclic AMP. Antioquine did not exert any inhibitory effect on the other forms of PDE, a cyclic GMP selective form (PDE V) and a low K_m cyclic AMP-PDE that is inhibited by cyclic GMP (CGI-PDE, PDE III).

7 The present work provides evidence that antioquine has properties both as a calcium entry blocker (possibly through the benzothiazepine recognition site in the calcium channel) and as a contractile agent. Its mechanism of action as a contractile agent is not related to Ca²⁺-entry and is hypothetically similar to that of calyculin-A or okadaic acid. The possible involvement of α -adrenoceptors in this paradoxical effect cannot be excluded. The rigidity of the molecule provides an interesting model for analyzing this contractile mechanism and the intracellular processes involved.

Keywords: Antioquine; α_1 -adrenoceptors; calcium antagonists; rat aorta; intracellular calcium

Introduction

Antioquine, a bisbenzyltetrahydroisoquinoline (BBTHIQ) alkaloid, was isolated from the stem bark of an endemic Colombian plant *Pseudoxandra sclerocarpa* (Cortes *et al.*, 1985; 1986). It is the main alkaloid from this species and is closely related structurally to tetrandrine (Figure 1), another BBTHIQ alkaloid isolated from *Stephania tetrandra*, that has been reported to possess effects on heart rate (*in vitro* and *in vivo*) and blood pressure, as does verapamil (Wang & Liu, 1985) which is consistent with tetrandrine being a calcium channel blocker (Wang & Liu, 1985; Fang & Jiang, 1986a,b; Yao *et al.*, 1987; Triggle *et al.*, 1989; Chen & Liu, 1990). Recently, other BBTHIQs, including dauricine (Dongke *et al.*, 1987) and (–)-daurisolone (Lu & Liu, 1990) have also been shown to have Ca²⁺ channel blocking properties.

It has been suggested that the mechanism of action of tetrandrine is related to an allosteric modulation of phenylalkylamine and 1,4-dihydropyridine binding, apparently

through interactions at the benzothiazepine receptor site at the L-channel (King *et al.*, 1988; Triggle *et al.*, 1989; Glossman *et al.*, 1991). Structurally, tetrandrine is unique among the known chemical classes of calcium entry blockers (Triggle *et al.*, 1989; Dong *et al.*, 1992). It is a dimer of two benzyloquinoline subunits condensed in a head to head, tail to tail fashion with 1S, 1'S, stereochemistry at the chiral isoquinoline carbons. Antioquine (Figure 1), shows a peculiar linkage by a biphenyl bond between the monomer BIQ rings (C11–C11') and the second bridge, a diaryl ether (C7–C7'), is more common in nature and it also appears in tetrandrine.

Preliminary studies (D'Ocon *et al.*, 1989; Ivorra *et al.*, 1992a) have shown that antioquine and several BBTHIQ alkaloids preferentially relax sustained contractions induced by KCl and induce a displacement to the right of the dose-response curves to CaCl₂ in rat isolated uterus. These results suggest that the relaxant effects of antioquine may be due to the blockade of calcium movements across the cell membrane in a similar way to the action of tetrandrine. The aim of the present study was to examine the mechanism of action of

¹ Author for correspondence.

Figure 1 Chemical structures of antioquine and tetrandrine.

antioquine by means of functional studies on rat aorta contracted by addition of noradrenaline, caffeine and KCl; radioligand binding studies using [^3H]-(+)-*cis*-diltiazem, [^3H]-nitrendipine, [^3H]-prazosin, and determination of inhibitory effects on cyclic nucleotide phosphodiesterase (PDE) activity.

We have examined the inhibitory effect of antioquine on the following cytosolic molecular forms of PDE isolated from bovine aorta: a PDE form called CaM-PDE, which preferentially hydrolyzes guanosine 3':5'-cyclic monophosphate (cyclic GMP) and is activated by calmodulin (CaM); a cyclic GMP selective form (cGMP-PDE) insensitive to the stimulatory effect of CaM; and two PDE forms, one belonging to the cyclic GMP-inhibited family (CGI-PDE, type III) and the other a rolipram-sensitive form (cAMP-PDE, type IV) (Lugnier *et al.*, 1986; Ivorra *et al.*, 1992b), which specifically hydrolyze adenosine 3':5'-cyclic monophosphate (cyclic AMP) with a low K_m and not stimulated by the addition of Ca^{2+} -CaM.

Methods

Functional study

Helically cut strips of thoracic aorta (denuded of endothelium) of male Wistar rats (200–220 g) were prepared and mounted as described by Furchgott & Zawadzki (1980). Each preparation was suspended in a 10 ml organ bath containing Krebs-bicarbonate solution (KBS), maintained at 37°C and gassed with 95% O_2 and 5% CO_2 . An initial load of 1 g was applied to each preparation and maintained throughout a 75–90 min equilibration period before agonist addition. Tension was recorded isometrically on a Phillips recorder (PM 8222) coupled to a Hewlett Packard amplifier (8805D) via force-displacement transducers (Gould Satham UC2).

A series of experiments was performed in KBS in order to determine if and to what extent antioquine induced a contractile response and/or relaxed rat aorta precontracted by noradrenaline or KCl. Contractions were expressed in mg (noradrenaline- or KCl-induced contractions) or as a percentage of the previous noradrenaline-induced contraction (antioquine-induced contractile response). Concentration-response curves of relaxation were obtained by the addition of cumulative concentrations of antioquine following the achievement of sustained contractions with noradrenaline (1 μM) or KCl (80 mM). Relaxations were expressed as a

percentage of the maximum tension obtained by agonist addition. E_{max} values representing the maximal relaxation obtained after addition of the highest dose of antioquine were calculated. The concentration needed to produce 50% inhibition (IC_{50}) was also calculated for each preparation from a least squares regression analysis of all points between 20–80% of the maximal response.

Figure 3a shows the experimental procedure used to study the action of antioquine on noradrenaline-induced contraction in the absence of extracellular calcium at 37°C or 25°C. In order to clarify the intracellular action of antioquine, similar experimental procedures were performed but noradrenaline was replaced by caffeine (Figure 3b). Caffeine yielded a contraction only at 25°C in Ca-free medium, so in protocols using caffeine as agonist, the temperature was decreased to 25°C.

After incubation for 1 h in KBS to stabilize the preparation, 1 μM noradrenaline or 10 mM caffeine were administered to contract the muscle, and the amplitude of the resultant contraction was monitored as a reference. The solution was then replaced by Ca^{2+} -free solution and left for 15 min before 1 μM noradrenaline or 10 mM caffeine was added and the contractile response monitored as a measure of the amount of Ca^{2+} released from the store sensitive to the agonist. After washing, a new addition of the agonist, noradrenaline or caffeine, did not elicit any contractile response. The solution was then replaced by normal KBS and left 20 min to refill the depleted Ca^{2+} stores. Noradrenaline or caffeine were again administered after 15 min in Ca^{2+} -free solution.

In rat aorta, contractile response to noradrenaline in Ca-free medium is associated with two different internal Ca -pools, one of which is common to caffeine (Noguera & D'Ocon, 1992). Further experiments were performed at 37°C and 25°C to test the effects of antioquine on Ca^{2+} release from the intracellular storage site sensitive only to noradrenaline. Figure 4 shows the experimental procedure used.

Contractions induced by antioquine in Ca^{2+} -free KBS were also obtained after addition of noradrenaline in Ca^{2+} -containing solution. The time of exposure to antioquine was 45 min in all cases. All contractile responses in Ca^{2+} -free medium were expressed as a percentage of the noradrenaline or caffeine-induced contraction in normal KBS.

Statistical analysis

Results are expressed as the mean $\pm$ s.e.mean of n preparations obtained from different animals. Statistical significance of differences between the means was assessed by Student's t test for unpaired data. P values of less than 0.05 were considered to represent significant differences.

Radioligand binding experiments

Binding of [^3H]-prazosin to rat cerebral cortex was saturable, reversible and showed high affinity, with a dissociation constant K_d of 0.14 nM (Schott *et al.*, 1988). [^3H]-nitrendipine and [^3H]-*cis*-diltiazem also bound to a single class of binding site in rat cortical membrane homogenate, with K_d values of 0.7 nM and 50 nM, respectively (Schaeffer *et al.*, 1988).

Membranes were prepared from cerebral cortex of male Wistar rats as previously described (Schott *et al.*, 1988). Briefly, the tissue was homogenized in 10 vol (w/v) of ice-cold buffer (50 mM Tris-HCl, pH 7.5) in an Ultra-turrax (twice, 15 s). The homogenate was centrifuged at 40,000 g at 4°C for 10 min and the resulting pellet was washed by resuspension and centrifugation under the same conditions. The final pellet was resuspended in incubation buffer to give a final protein concentration of about 1 mg ml^{-1} . Membrane aliquots (250 μl) were incubated in a final volume of 500 μl in 50 mM Tris-HCl (pH 7.5) with [^3H]-prazosin (0.1–0.2 nM) or [^3H]-nitrendipine (0.3–0.4 nM) or in 50 mM Tris-HCl pH 7.5 containing 1 mg ml^{-1} of bovine serum albumin (BSA) with [^3H]-

(+)-*cis*-diltiazem (3–4 nM), in the absence or in the presence of drugs at various concentrations.

Incubations were carried out at 25°C for 30 min ($[^3\text{H}]$ -prazosin), 90 min ($[^3\text{H}]$ -nitrendipine) or 120 min ($[^3\text{H}]$ -(+)-*cis*-diltiazem), after which the reaction was terminated by the addition of 3.0 ml ice-cold buffer and rapid filtration over Whatman GF/B filters that were washed with 9 ml (3×3 ml) of ice-cold buffer. For $[^3\text{H}]$ -(+)-*cis*-diltiazem binding experiments, filters were pretreated for 120 min with polyethyleneimine 0.3%. The radioactivity bound to the filters was determined by liquid scintillation counting. Non-specific binding was determined in the presence of 10^{-6} M phentolamine, 10^{-6} M nifedipine or 10^{-5} M diltiazem, respectively. All experiments were performed in triplicate.

Displacement curves were analyzed by a computerized non-linear curve-fitting program and K_i values were calculated from the formula of Cheng & Prusoff (1973).

Phosphodiesterase inhibition

Cytosolic cyclic nucleotide phosphodiesterase activities were isolated from the media layer of bovine aorta by a modification of the methods of Lugnier *et al.* (1986). Briefly, the tissue was homogenized in 10 vol (w/v) isotonic buffer A (25 mM sodium phosphate pH 6.6, 250 mM saccharose, 2 mM magnesium acetate (MgAc), 1 mM dithiothreitol, 5 mM EGTA, 2000 u ml $^{-1}$ aprotinin, 10 μg ml $^{-1}$ soya bean trypsin inhibitor and 10 μg ml $^{-1}$ leupeptin) using an Ultra-turrax (six times, 10 s) and a glass pestle homogenizer, and then centrifuged at 105,000 *g* for 60 min. The resulting supernatant fraction (40 ml) was applied to a DEAE-Sephacel ion-exchange column (15 cm $\times$ 1.5 cm) and first eluted by buffer B (25 mM phosphate pH 6.6, MgAc 2 mM and dithiothreitol 1 mM) until no more absorbance was detected in the eluate at 280 nm. Elution was then continued with a linear gradient of 0–0.55 M of NaCl in buffer B (flow rate 25 ml h $^{-1}$). Each fraction was tested for PDE activity.

Since DEAE-Sephacel did not allow the separation of CaM-PDE from cGMP-PDE, as did DEAE-Trisacryl chromatography (Lugnier *et al.*, 1986) a further higher performance liquid chromatography (h.p.l.c.) repurification was carried out. The first peak was then injected into an h.p.l.c. column (TSK-DEAE-5PW), washed for 20 min with elution buffer (25 mM phosphate pH 6.6) and eluted (0.8 ml min $^{-1}$) by a linear NaCl gradient (0.05–0.3 M) in elution buffer. Fractions under each PDE activity peak were pooled, dialyzed against buffer (20 mM Tris-HCl, 2 mM MgAc pH 7.5) and stored in aliquots at -80°C with bovine serum albumin (BSA).

PDE activities were measured by the two-step assay described by Wells *et al.* (1975) at a substrate ($[^3\text{H}]$ -cyclic AMP or $[^3\text{H}]$ -cyclic GMP) concentration of 1 μM , in the following buffer: 50 mM Tris-HCl pH 7.5, 2 mM MgAc, 1 mg ml $^{-1}$ BSA and in the presence of 10 μM CaCl $_2$ and 18 nM calmodulin (CaM) or in the absence of calcium and CaM but with 1 mM EGTA. To prevent the influence of cross-contamination between CGI-PDE (PDE III) and cAMP-PDE (PDE IV), the studies performed with these forms were always carried out in the presence of 100 μM rolipram or 100 μM cyclic GMP, respectively.

The IC $_{50}$ (concentration which produced 50% inhibition of substrate hydrolysis) for the compounds studied was determined from the concentration-response curves obtained with five concentrations of inhibitor and calculated by a non-linear regression. The apparent K_i values were obtained according to Cheng & Prusoff (1973). Results are expressed as mean $\pm$ s.e.mean of three determinations made in duplicate with three different enzymatic preparations.

Drugs and solutions

The following drugs were used: (–)-noradrenaline L-tartrate and acetylcholine (Merck; Darmstadt, FRG); nifedipine, dil-

tiazem, prazosin, papaverine, caffeine, cyclic AMP, cyclic GMP and phentolamine (Sigma Chemical Co.; St. Louis, U.S.A.); rolipram (Schering AG). Antioquine was isolated by the method of Cortes *et al.* (1985) from *Pseudoxandra sclerocarpa* (Annonaceae).

The composition of KBS was as follows (mM): NaCl 118, KCl 4.75, CaCl $_2$ 1.8, MgCl $_2$ 1.2, KH $_2$ PO $_4$ 1.2, NaHCO $_3$ 25 and glucose 11. Ca-free solution had the same composition except that CaCl $_2$ was omitted and EDTA (0.1 mM) was added.

$[^3\text{H}]$ -prazosin (20.2 Ci mmol $^{-1}$), $[^3\text{H}]$ -nitrendipine (70 Ci mmol $^{-1}$), [^3H]-cyclic AMP (30–50 Ci mmol $^{-1}$) and [^3H]-cyclic GMP (5–15 Ci mmol $^{-1}$) were purchased from New England Nuclear. $[^3\text{H}]$ -(+)-*cis*-diltiazem (154 Ci mmol $^{-1}$) was obtained from Amersham. Other reagents were of analytical grade.

Caffeine was dissolved in Ca $^{2+}$ -free KBS. Nifedipine was dissolved in ethanol (10^{-2} M) before being diluted in distilled water. The other drugs were dissolved in distilled water. All solutions were prepared daily and pH adjusted to 7.0.

Results

Vasorelaxant response to antioquine

The contractile responses of rat aorta elicited by depolarizing solution (KCl 80 mM) at 37°C or 25°C, were 600.8 ± 48.8 mg ($n = 10$) and 638.3 ± 97.8 mg ($n = 6$) respectively. The same contraction elicited in the presence of phentolamine 10^{-5} M at 37°C, was lower (358.3 ± 33.7 mg, 59.7% of the first one).

Antioquine (0.1–300 μM) produced concentration-dependent relaxations of KCl-depolarized rat aorta at 37°C both in the presence and absence of phentolamine. Experiments performed at 25°C gave similar results (Table 1).

Noradrenaline (1 μM) produced a sustained contraction of rat isolated thoracic aorta incubated in KBS at 37°C (584.2 ± 45.2 mg, $n = 10$) and a similar contraction at 25°C (623.3 ± 86.4 mg, $n = 10$). This dose of noradrenaline has proved to be the maximal for contraction in rat aorta (Noguera & D'Ocon, 1992).

Addition of cumulative concentrations of antioquine (0.1–300 μM) on the sustained contraction induced by noradrenaline 1 μM in KBS induced concentration-dependent relaxations. The E_{max} and IC $_{50}$ values are shown in Table 1. No differences were observed at 37°C or 25°C.

Vasoconstrictor response to antioquine

Noradrenaline (1 μM) produced a sustained contraction in rat isolated thoracic aorta incubated in KBS (see above). After washing, subsequent addition of antioquine 30 μM did not modify the basal tone in KBS or Ca $^{2+}$ -free KBS at 37°C or 25°C. A higher concentration of this alkaloid (300 μM) induced a sustained contractile response in the presence ($61.6 \pm 2.2\%$, with respect to noradrenaline-induced contraction in KBS, $n = 6$) or absence ($61.0 \pm 1.7\%$, $n = 4$) of extracellular Ca $^{2+}$, at 37°C (Figure 2). This contractile response

Table 1 Inhibitory potencies of antioquine on contractions induced by noradrenaline, KCl and KCl + phentolamine 10^{-5} M (Phen) at 37°C or 25°C

Temperature	Noradrenaline	KCl	KCl + Phen
37°C E_{max} (%)	46.7 ± 2.7 $n = 14$	63.0 ± 3.7 $n = 10$	63.3 ± 4.7 $n = 6$
IC $_{50}$ (μM)	> 300	185.1 ± 53.9	187.0 ± 65.0
25°C E_{max} (%)	50.8 ± 3.2 $n = 6$	66.4 ± 6.7 $n = 6$	
IC $_{50}$ (μM)	> 300	154.1 ± 25.3	

Values are mean $\pm$ s.e.mean; n = number of experiments.

Figure 2 Typical recording of the effects of 300 μ M antioquine (ATQ) on rat isolated aorta in the presence or absence of calcium. NA: 1 μ M noradrenaline; W: wash.

was significantly less ($P < 0.002$) when the experiments were carried out at 25°C ($26.0 \pm 4.8\%$, $n = 9$ in the presence and $24.3 \pm 4.2\%$ in the absence, of extracellular Ca^{2+}).

Prazosin, nifedipine and diltiazem did not inhibit the contractile response to antioquine in the presence or absence of calcium (Table 2). Moreover, prazosin and nifedipine slightly increased the contraction in the presence of extracellular calcium. Papaverine partially inhibited the contractile response to antioquine under both experimental conditions (Table 2).

Effect of antioquine on noradrenaline- or caffeine-induced contractions in a Ca^{2+} -free medium

After 15 min in Ca^{2+} -free solution, addition of noradrenaline 1 μ M induced a phasic contraction followed by a tonic one. However, after washing, the contraction was not evoked upon the second application of noradrenaline in Ca^{2+} -free solution. After a 20 min resting period in KBS, the application of noradrenaline after 15 min in Ca^{2+} -free solution induced a contraction similar in magnitude to that produced by its first application, thereby indicating that loading 1.8 mM Ca^{2+} for 20 min had refilled the calcium storage sites.

Addition of antioquine (300 μ M) 10 min before addition of noradrenaline, elicited a slowly developed and sustained contraction ($14.15 \pm 2.06\%$, $n = 7$, with respect to noradrenaline-induced contraction in KBS) at 37°C, but no contractile response to antioquine was observed at 25°C. Subsequent addition of noradrenaline in the presence of antioquine did not modify the profile of the antioquine-induced contraction at 37°C. This response was similar to that obtained when

Table 2 Effects of different agents on the contractile response induced by antioquine (300 μ M) in KBS and Ca^{2+} -free solution

Agent	Concentration	% contraction in KBS	% contraction in Ca^{2+} -free solution
Control		61.6 ± 2.2 $n = 6$	61.0 ± 1.7 $n = 4$
Prazosin	0.1 μ M	$83.5 \pm 3.8^*$ $n = 6$	64.2 ± 4.3 $n = 5$
Nifedipine	1 μ M	$72.8 \pm 3.7^*$ $n = 9$	52.7 ± 4.3 $n = 6$
Diltiazem	100 μ M	64.0 ± 3.9 $n = 6$	66.8 ± 7.7 $n = 3$
Papaverine	100 μ M	$32.2 \pm 5.1^*$ $n = 4$	$21.3 \pm 1.6^*$ $n = 4$

The concentrations of prazosin, nifedipine, diltiazem and papaverine completely inhibited noradrenaline- or KCl-induced contraction in KBS.

% contractions are calculated from the initial contraction by noradrenaline in Krebs solution.

Values are mean $\pm$ s.e.mean; n = number of experiments.

* $P < 0.002$ compared with control.

only antioquine (300 μ M) was added to Ca^{2+} -free or Ca^{2+} -containing medium. The alkaloid did not modify the contractile response to noradrenaline at 25°C (Table 3).

At 25°C, caffeine 10 mM induced a rapid transient contraction in Ca^{2+} 1.8 mM solution (Table 3, Figure 3b). Addition of caffeine after 15 min of exposure to a Ca^{2+} -free solution yielded a phasic contraction. After washing, addition of caffeine did not induce any contractile response. After a loading period (20 min) in KBS to refill the intracellular Ca^{2+} -stores, the caffeine-induced contraction in Ca^{2+} -free medium (15 min) was restored. Preincubation (10 min) with antioquine (300 μ M) increased the phasic contractile response induced by caffeine in Ca^{2+} -free medium after refilling of the intracellular Ca^{2+} stores (Table 3).

Table 3 Effect of antioquine 300 μ M (ATQ) on phasic contraction of rat aorta induced by noradrenaline or caffeine in Ca^{2+} -free medium (see Figures 3a and b)

Agonist (A)	Temperature ($^{\circ}\text{C}$)	A_1 (%)	$A_2 + \text{ATQ}$ (%)	n
NA	37 $^{\circ}$	22.0 ± 1.8	$51.1 \pm 2.6^{**}$	7
NA	25 $^{\circ}$	26.4 ± 3.5	24.1 ± 2.7	6
Caff	25 $^{\circ}$	27.5 ± 5.0	$48.1 \pm 5.4^*$	5

% are calculated from the initial contraction by noradrenaline (NA) or caffeine (Caff) in Krebs solution. Values are mean $\pm$ s.e.mean; n = number of experiments.

* $P < 0.005$ compared with A_1 .

[†]Sustained contraction after 45 min of exposure to antioquine.

A_1 : addition of agonist after 15 min in Ca^{2+} -free medium.

A_2 : addition of agonist after depletion, complete refilling of internal Ca^{2+} -stores and subsequent loading for 15 min in Ca^{2+} -free medium.

Figure 3 Schematic representation of the effect of antioquine on noradrenaline (NA)-induced contraction (a) or caffeine (Caff)-induced contraction (b) in the absence of extracellular calcium. NA_1 or Caff_1 : addition of the agonist after 15 min incubation in Ca^{2+} -free medium. NA_2 or Caff_2 : second addition of the agonist after washing (W) in Ca^{2+} -free medium. ATQ : addition of antioquine (300 μ M) after a 15 min resting period in KBS (Ca^{2+} 1.8 mM) and 20 min in Ca^{2+} -free solution.

Addition of noradrenaline (1 μ M) in Ca^{2+} -free medium (NA_1 , Figure 4) elicited a contractile response and depleted intracellular stores sensitive to this agonist. A refilling of these internal Ca^{2+} -pools was obtained by subsequent loading in Ca^{2+} -containing solution (20 min). After 15 min in Ca^{2+} -free medium, application of caffeine (10 mM) evoked a transient contractile response at 25°C but not at 37°C (Caff_1 , Figure 4). No contraction was evoked when caffeine was added a second time (Caff_2 , Figure 4), which suggests that the intracellular Ca^{2+} -stores sensitive to caffeine were emptied. After washing, addition of noradrenaline (NA_2 , Figure 4) in Ca^{2+} -free medium induced a small and tonic contractile response at 25°C and 37°C in the absence of antioquine, and a similar response at 25°C in the presence of antioquine (Table 4). At 37°C, addition of antioquine in Ca^{2+} -free medium, induced a contractile response and addition of noradrenaline (10 min later) did not modify this contraction with respect to that obtained when only antioquine was added in Ca^{2+} -free medium (Table 4).

Binding assays

The interactions of antioquine with [^3H]-prazosin and [^3H]-(+)-*cis*-diltiazem binding are shown in Figure 5. Antioquine fully inhibited [^3H]-prazosin binding to cortical membranes, with an inhibition constant of $0.90 \pm 0.34 \mu\text{M}$. The fact that the pseudo-Hill coefficient (slope factor) was not significantly different from unity ($n_H = 0.98 \pm 0.07$) suggests direct competition between antioquine and the radioligand for a single common binding site. In addition, antioquine inhibited bin-

Figure 4 Experimental procedure used to study the effect of antioquine in the absence of extracellular calcium on noradrenaline (NA)-induced contraction after depletion of caffeine (Caff)-sensitive intracellular calcium pools. NA_1 : addition of 1 μM noradrenaline after 15 min incubation in Ca^{2+} -free medium. NA_2 : second addition of the agonist after washing (W) in Ca^{2+} -free medium. Caff_1 : addition of 10 mM caffeine after a 15 min resting period in KBS (Ca^{2+} 1.8 mM) and 20 min in Ca^{2+} -free solution. Caff_2 : second addition of the agonist after washing (W) in Ca^{2+} -free medium. NA_2 : addition of noradrenaline in the presence of antioquine (ATQ) 300 μM , after depletion of caffeine-sensitive intracellular calcium stores and washing (W) in Ca^{2+} -free solution.

Table 4 Influence of temperature on contractile responses to noradrenaline (NA) and caffeine (Caff) in Ca-free solution in presence (b) and absence (a) of antioquine 300 μM (ATQ) (see Figure 4)

Temperature	n	NA_1	Caff	ATQ	NA_2
(a) 37°C	7	21.6 ± 3.2	NR	—	7.3 ± 0.8
(b) 37°C	4	19.5 ± 3.6	NR	13.8 ± 3.0	62.8 ± 4.3
(a) 25°C	6	28.1 ± 3.5	12.3 ± 2.1	—	8.6 ± 0.7
(b) 25°C	5	26.3 ± 4.8	13.3 ± 3.1	NR	9.7 ± 3.5

% contractions are calculated from the initial contraction by noradrenaline in Krebs solution. Values are mean $\pm$ s.e.mean; n = number of experiments. NR = no response.

Figure 5 Displacement curves of [^3H]-prazosin (O) or [^3H]-(+)-*cis*-diltiazem specific binding (●) by antioquine. Each point is the mean (with s.e.mean) from three experiments made in triplicate.

ding of [^3H]-(+)-*cis*-diltiazem but with a lower potency ($K_i = 20.58 \pm 4.39 \mu\text{M}$) and with a similar pseudo-Hill slope ($n_H = 0.89 \pm 0.19$). This suggests that the interaction between antioquine and [^3H]-(+)-*cis*-diltiazem is also a simple competition for a single common binding site.

[^3H]-nitrendipine binding was not affected by antioquine in concentrations up to 100 μM (results not shown).

Inhibition of bovine aorta cyclic nucleotide phosphodiesterase enzymes

As shown in Table 5, antioquine has a weak inhibitory effect on PDE I and IV forms but did not have a significant action on the other forms (PDE III and V). In contrast, papaverine exerted a relatively non-selective inhibitory effect on all PDE forms, although the inhibition of the calmodulin-sensitive form (PDE I) appeared weaker than that of the other forms.

Discussion

The present experiments have demonstrated that antioquine, an alkaloid isolated from *Pseudoxandra sclerocarpa*, has a dual effect on the contractile mechanism of rat aorta: lower concentrations inhibit contractile responses whereas the highest concentration tested elicited a contraction that is not dependent on extracellular calcium.

Antioquine inhibited the contractile responses of rat aorta to the adrenoceptor agonist, noradrenaline, and high-potassium depolarization at 37°C and 25°C in the absence of vascular endothelium. Therefore, the vasorelaxation caused by antioquine was not mediated by either endothelium-derived relaxing factor (EDRF) or prostacyclin (PGI_2) (Jaffe, 1985; Vanhoutte *et al.*, 1986).

Table 5 Effects of agents on cyclic nucleotide phosphodiesterases (PDE) isolated from bovine aorta

PDE activity	³ H substrate	K _i (μM)	
		Antioquine	Papaverine*
CaM-PDE type I	Cyclic GMP + EGTA	97.4	8
	Cyclic GMP + CaM	45.8 ± 3.0	27.5 ± 2.2
cGMP-PDE type V	Cyclic GMP + EGTA	> 300	1.08 ± 0.36
CGI-PDE type III	Cyclic AMP + EGTA	> 300	0.76
cAMP-PDE type IV	Cyclic AMP + EGTA	62.3 ± 12.7	1.68

Data shown are mean ± s.e.mean from three determinations obtained in different enzymatic preparations. See text for abbreviations.

*Data from Ivorra *et al.* (1992c).

In order to determine to what extent inhibition of PDE is involved in the vasorelaxant response to antioquine, we tested the effect of this compound on PDE activities isolated from bovine aorta as a model for vascular smooth muscle. Our results show that antioquine did not exhibit any potent inhibitory action on the different PDE forms. It only weakly inhibits CaM-PDE (PDE I) and cAMP-PDE (PDE IV), and unlike papaverine, has no inhibitory effect on the other forms (PDEs III and V). Therefore, it is probable that inhibition of some PDE form is not a major mechanism responsible for the relaxant response to antioquine.

Analysis of the results obtained in the binding assays indicates that antioquine inhibited binding of [³H]-(+)-*cis*-diltiazem and [³H]-prazosin with a similar pseudo-Hill slope (0.89 ± 0.19 and 0.98 ± 0.07 respectively). This suggests that the interaction between antioquine and [³H]-(+)-*cis*-diltiazem or [³H]-prazosin is a simple competition for a single common binding site. These results could suggest a direct action of antioquine at α₁-adrenoceptors and/or the benzothiazepine receptor site at the calcium channel.

The fact that antioquine relaxes rat aorta may be interpreted as an antagonistic action with respect to a noradrenaline- or KCl-induced contraction. Analysis of the IC₅₀ values of concentration-response curves of relaxation indicates that antioquine exhibits greater activity on the contractile response induced by KCl. Thus, the relaxant effects of antioquine may be due to the blockade of calcium movements through potential operated channels. The fact that the IC₅₀ of antioquine on the contractile response induced by KCl was similar in the presence and absence of phentolamine indicates that the relaxant action shown by this alkaloid is not dependent on α-adrenoceptor blocking activity.

Results were different when antioquine was added at 37°C at the higher concentration tested (300 μM). In this case, antioquine induced a slow-developing sustained contraction in both Ca²⁺-containing and Ca²⁺-free medium. The magnitude of this contraction after 45 min of exposure to antioquine was about 60% with respect to that of noradrenaline in Ca²⁺-containing solution. The contractile response was similar both in the presence and absence of extracellular calcium but was clearly attenuated at 25°C. Therefore, it is mediated by a temperature-dependent process. This contraction was reduced by papaverine (with and without calcium in the medium); and, as expected, contractile responses to antioquine were not modified by the presence of calcium antagonists (nifedipine and diltiazem), the absence of external calcium, or prazosin. Paradoxically, nifedipine and prazosin increased contractions in Ca²⁺-containing solution.

Thus, our present results showed that the antioquine-induced contraction was not mediated by α₁-adrenoceptor activation or entry of external cations through voltage-operated channels. These findings also rule out the possibility of an involvement of noradrenaline release from perivascular adrenergic nerve endings. We suggest that this contraction is more likely due to an intracellular action of antioquine. The fact that prazosin and nifedipine, in Ca-containing solution, increase this contractile response to antioquine means that a possible participation of α-adrenoceptors or voltage-dependent calcium channels cannot be excluded. Moreover,

the evidence that only papaverine inhibits the antioquine-induced contractions confirms the peculiarity of this process and suggests an intracellular mechanism related to the contractile apparatus.

In order to examine the possible intracellular action of antioquine, functional studies were performed in Ca²⁺-free medium and noradrenaline- or caffeine-induced contractile responses were elicited in the presence or absence of antioquine. The results obtained demonstrated that in rat isolated aorta, at 25°C, antioquine did not elicit any contractile response, did not modify the contraction induced by noradrenaline and increased caffeine-induced contraction. At 37°C, antioquine induced a contractile response in all the experimental procedures used and previous addition of caffeine or subsequent addition of noradrenaline did not modify this contraction.

Recent studies have shown that the alkaloid, ryanodine, has a contractile action on smooth muscle cells, selectively acts on the Ca²⁺ channel in the sarcoplasmic reticulum membrane when it is in the open state and depletes the Ca²⁺ in both caffeine- and noradrenaline-sensitive stores (Meissner, 1986; Herrman-Frank *et al.*, 1991). The compound behaves as a functional Ca²⁺ store blocker and inhibits contractile responses to caffeine and noradrenaline in Ca²⁺-free medium (Sato *et al.*, 1988; Hisayama *et al.*, 1990). It, therefore, seems clear that antioquine and ryanodine do not act by a common mechanism.

Antioquine-evoked contraction in Ca²⁺-free medium may be induced by intracellular Ca²⁺ release from noradrenaline- and caffeine-insensitive sources; alternatively this contraction may not be due to an intracellular Ca²⁺ mobilization but to a direct action on the contractile machinery.

A possible source of intracellular Ca²⁺ responsible for antioquine-induced contraction could be the mitochondria. Autoradiographic and electron microscopic studies have confirmed that diltiazem, verapamil and dihydropyridines all accumulate within the mitochondrial matrix (Sato *et al.*, 1971; Nakajima *et al.*, 1975; Lullman *et al.*, 1979; Pang & Sperelakis, 1983; Tagami *et al.*, 1985) and have the ability to alter Ca²⁺ transport into the mitochondria. These findings support the hypothesis that antioquine, which binds selectively to the benzothiazepine receptor site at the plasma membrane calcium channel, also acts in the same way at the mitochondrial membrane. It would then inhibit Ca²⁺ uptake and the capacity of mitochondria to regulate the intracellular free Ca²⁺ level.

Another explanation for the present findings is a direct sensitization of the contractile machinery to Ca²⁺. This sensitization could result from an action on the contractile proteins *via* an effect on protein kinases, as occurs with phorbol esters and endothelin-1 (Salaices *et al.*, 1990; Itoh *et al.*, 1991), or *via* phosphatase inhibition. With respect to the latter mechanism, it should be noted that two known potent inhibitors of type 1 and type 2A phosphatases, okadaic acid and calyculin-A (Ishihara *et al.*, 1989; Obara *et al.*, 1989), induce a similar contraction of smooth muscle, which is also independent of extracellular calcium. Moreover, Karaki *et al.* (1989) demonstrated that okadaic acid at concentrations lower than that needed to induce a contractile response, has

an inhibitory effect on smooth muscle contraction, as does antioquine. In any case, further work will be required to establish the exact mechanism of the antioquine-elicited contraction in rat aorta.

Structurally, antioquine is closely related to tetrandrine and, as stated earlier, tetrandrine is a calcium channel blocker that interacts at the benzothiazepine receptor site at the L-channel. However, antioquine and tetrandrine appear to act in different ways at the intracellular level: tetrandrine does not elicit any contractile response and inhibits noradrenaline-induced contraction in Ca^{2+} -free medium (unpublished results). The different behaviour shown by the two alkaloids can be explained on the basis of their different structural features. The structural modifications found in antioquine with respect to tetrandrine are the presence of an unusual linkage by a biphenyl bond between the monomer BIQ rings (C_{11} – $\text{C}_{11'}$) and the absolute configuration at the chiral centres (1S,1'R antioquine and 1S,1'S tetrandrine). In the benzyloisoquinoline group containing the chiral carbon with absolute configuration 1S, the phenyl ring must remain orthogonal to the plane defined by the isoquinoline system. In contrast, in the benzyloisoquinoline containing the chiral

carbon with absolute configuration 1R, the phenyl and the heterocyclic rings are nearly coplanar. These different topological characteristics could determine the different behaviour of the two alkaloids.

Only compounds such as antioquine have a temperature-dependent contractile activity of apparent intracellular origin. This means that antioquine could be an interesting compound for studying this process, due to the presence of the biphenyl linkage that restricts conformational mobility in this molecule.

In summary, these data show that antioquine can relax smooth muscle cells in the rat aorta previously contracted by noradrenaline or KCl, probably due to activity as a calcium antagonist. However, at the highest concentration tested, it elicits a slow-developing and sustained contraction independent of extracellular calcium and is not inhibited by prazosin, nifedipine or diltiazem. Antioquine exhibits an affinity for [^3H]-prazosin and [^3H]-(+)-*cis*-diltiazem binding sites and selectively inhibits, although weakly, some PDE forms (PDE I and IV) isolated from bovine aorta; however, the mechanism for its contractile action requires further investigation.

References

- CHEN, S.H. & LIU, G.Z. (1990). Calcium antagonists from traditional chinese herbs. *Drug New Perspectives*, **3**, 425–428.
- CHENG, Y.G. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.
- CORTES, D., SAEZ, J., HOCQUEMILLER, R., CAVE, A. & CAVE, A. (1985). Alcaloides de Annonacées: alcaloides du *Pseudoxandra off. lucida*: étude de l'antioquine et de ses dérivés. *J. Nat. Prod.*, **48**, 76–85.
- CORTES, D., HOCQUEMILLER, R., CAVE, A. & SAEZ, J. (1986). Annonaceae alkaloids. Part 64. Minor alkaloids from the bark of *Pseudoxandra sclerocarpa*. *J. Nat. Prod.*, **49**, 854–858.
- D'OCON, M.P., CANDENAS, M.L., ANSELM, E., ZAFRA-POLO, M.C. & CORTES, D. (1989). Antioquine: a new bisbenzyloisoquinoline alkaloid with calcium antagonist activity. *Arch. Int. Pharmacodyn. Ther.*, **297**, 205–216.
- DONG, H., LEE, C.M., HUANG, W.L. & PENG, S.X. (1992). Cardiovascular effects of substituted tetrahydroisoquinolines in rats. *Br. J. Pharmacol.*, **107**, 262–268.
- DONGKE, Z., GENG, S.Z. & PEI, L.Q. (1987). Effect of dauricine on the auricle of guinea pig. *Chin. Pharmacol. Bull.*, **3**, 100–103.
- FANG, D. & JIANG, M. (1986a). Studies on tetrandrine calcium antagonistic action. *Chin. Med. J.*, **99**, 638–644.
- FANG, D. & JIANG, M. (1986b). A new calcium antagonist of chinese medicinal origin: tetrandrine. *J. Hypertens.*, **4** (suppl. 6), S150–S152.
- FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.
- GLOSSMAN, H., ZECH, C., STRIESSING, J., STAUDINGER, R., HALL, L., GREENBERG, R. & ARMAH, B.I. (1991). Very high affinity interaction of DPI 201–106 and BDF 8784 enantiomers with the phenylalkylamine sensitive Ca^{2+} channel in *Drosophila* head membranes. *Br. J. Pharmacol.*, **102**, 446–452.
- HERRMANN-FRANK, A., DARLING, E. & MEISSNER, G. (1991). Functional characterization of the Ca^{2+} release channel of vascular smooth muscle sarcoplasmic reticulum. *Pflügers Arch.*, **418**, 353–359.
- HISAYAMA, T., TAKAYANAGI, I. & OKAMOTO, Y. (1990). Ryanodine reveals multiple contractile and relaxant mechanisms in vascular smooth muscle: simultaneous measurements of mechanical activity and of cytoplasmic free Ca^{2+} level with fura-2. *Br. J. Pharmacol.*, **100**, 677–684.
- ISHIHARA, H., OZAKI, H., SATO, K., MASATOSHI, H., KARAK, H., WATAB, S., KATO, Y., FUSEYANI, N., HASHIMOTO, K., VEMURA, D. & HARTSHORNE, D.J. (1989). Calcium-independent activation of contractile apparatus in smooth muscle by calyculin-A. *J. Pharmacol. Exp. Ther.*, **250**, 388–396.
- ITO, H., HIGUCHI, H., HIRAOKA, N., ITO, M., KONICHI, T., NAKANO, T. & LEDERIS, K. (1991). Contraction of rat thoracic aorta strips by endothelin-1 in the absence of extracellular Ca^{2+} . *Br. J. Pharmacol.*, **104**, 847–852.
- IVORRA, M.D., CERCOS, A.C., ZAFRA-POLO, M.C., PEREZ-PRIETO, J., SAEZ, J., CORTES, D. & D'OCON, M.P. (1992a). Selective chiral inhibition of calcium entry promoted by bisbenzyloisoquinolines in rat uterus. *Eur. J. Pharmacol.*, **219**, 303–309.
- IVORRA, M.D., LE BEC, A. & LUGNIER, C. (1992b). Characterization of membrane-bound cyclic nucleotide phosphodiesterases from bovine aortic smooth muscle. *J. Cardiovasc. Pharmacol.*, **19**, 532–540.
- IVORRA, M.D., LUGNIER, C., SCHOTT, C., CATRET, M., NOGUERA, M.A., ANSELM, E. & D'OCON, P. (1992c). Multiple actions of glaucine on cyclic nucleotide phosphodiesterases, α_1 -adrenoceptor and benzothiazepine binding site at the calcium channel. *Br. J. Pharmacol.*, **106**, 387–394.
- JAFFE, E.A. (1985). Physiological function of normal endothelial cells. *Ann. N.Y. Acad. Sci.*, **454**, 279–291.
- KARAKI, H., MITSUI, M., NAGASE, H., OZAKI, H., SHIBATA, S. & UEMURA, D. (1989). Inhibitory effect of a toxin okadaic acid, isolated from the black sponge on smooth muscle and platelets. *Br. J. Pharmacol.*, **98**, 590–596.
- KING, V.F., GARCIA, M.L., HIMMEL, D., REUBEN, J.P., LAM, Y.T., PAN, J., HAN, G. & KACZOROWSKI, G.J. (1988). Interaction of tetrandrine with slowly inactivating calcium channels. *J. Biol. Chem.*, **263**, 2238–2244.
- LU, M.Y. & LIU, G.Q. (1990). The effects of (–)-daurisolone on Ca^{2+} influx in presynaptic nerve terminals. *Br. J. Pharmacol.*, **101**, 45–48.
- LUGNIER, C., SCHOEFFTER, P., LE BEC, A., STROUTHON, E. & STOCLET, J.C. (1986). Selective inhibition of cyclic nucleotide phosphodiesterases of human, bovine and rat aorta. *Biochem. Pharmacol.*, **35**, 1743–1751.
- LULLMANN, H.M., TIMMERMANS, P.B. & ZIEGLER, A. (1979). Accumulation of drugs by resting or beating cardiac tissues. *Eur. J. Pharmacol.*, **60**, 277–285.
- MEISSNER, G. (1986). Ryanodine activation and inhibition of the Ca^{2+} release channel of sarcoplasmic reticulum. *J. Biol. Chem.*, **261**, 6300–6306.
- NAKAJIMA, H., HOSHIYAMA, M., YAMASHITA, K. & KIYOMOTO, A. (1975). Effect of diltiazem on electrical and mechanical activity of isolated cardiac ventricular muscle of guinea-pig. *Jpn. J. Pharmacol.*, **25**, 383–392.
- NOGUERA, M.A. & D'OCON, M.P. (1992). Different and common intracellular calcium-stores mobilized by noradrenaline and caffeine in vascular smooth muscle. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 333–341.
- OBARA, K., TAKAI, A., RUEGG, J.C. & DE LANEROLLE, P. (1989). Okadaic acid, a phosphatase inhibitor, produces a Ca^{2+} and calmodulin-independent contraction of smooth muscle. *Pflügers Arch.*, **414**, 134–138.
- PANG, D.C. & SPERELAKIS, N. (1983). Uptake of [^3H]-nitrendipine into cardiac and smooth muscle. *Biochem. Pharmacol.*, **32**, 1660–1663.

- SALAIRES, M., BALFAGON, G., ARRIBAS, S., DE SAGARRA, M. & MARIN, J. (1990). Effects of phorbol 12,13-dibutyrate on the vascular tone and on norepinephrine- and potassium- induced contractions of cat cerebral arteries. *J. Pharmacol. Exp. Ther.*, **255**, 66–73.
- SATO, M., NAGAO, T., YAMAGUCHI, I., NAKAJIMA, H. & KIYOMOTO, A. (1971). Pharmacological studies on a new 1,5-benzothiazepine derivative (CRD-401 = Diltiazem). *Arzneim. Forsch. Drug. Res.*, **21**, 1338–1343.
- SATO, K., OZABE, H. & KARAKI, H. (1988). Multiple effects of caffeine on contractions and cytosolic free Ca levels in vascular smooth muscle of rat aorta. *Naunyn-Schmied. Arch. Pharmacol.*, **333**, 443–448.
- SCHAEFFER, P., LUGNIER, C. & STOCLET, J.C. (1988). Interaction of calmodulin and calcium antagonist with [³H]-diltiazem and [³H]-nitrendipine binding sites. *J. Cardiovasc. Pharmacol.*, **12**, (Suppl. 4) S102–S103.
- SCHOTT, C., TETSI, L., HEITZ, C., STAMBACH, J.F., JUNG, L. & STOCLET, J.C. (1988). Stereoselective blockade of α -adrenoceptors by berbine derivatives. *Arzneim-Forsch. Drug Res.*, **38**, 1567–1571.
- TAGAMI, M., NARA, Y., KUBOTA, A., SUNAGA, T., MAELZAWA, H., HORIE, R. & YAMORI, Y. (1985). Electromicroscopic autoradiographic study of the distribution of [³H]-diltiazem in myocardial cells. *Jpn. Heart J.*, **65**, 823–832.
- TRIGGLE, D.J., LANGS, D.A. & JANIS, R.A. (1989). Ca-channels ligands: structure-function relationships of the 1,4-dihydropyridines. *Med. Res. Rev.*, **9**, 123–180.
- VANHOUTTE, P.M., RUBANY, G.M., MELLER, U.M. & HOUSTON, D.S. (1986). Modulation of vascular smooth muscle contraction by endothelium. *Annu. Rev. Physiol.*, **48**, 307–320.
- WANG, Z.G. & LIU, G.Z. (1985). Advances in natural products in China. *Trends Pharmacol. Sci.*, **6**, 423D–426D.
- WELLS, J.W., BAIRD, C.E., WU, V.J. & HARDMAN, J.C. (1975). Cyclic nucleotide phosphodiesterase activity of pig coronary arteries. *Biochem. Biophys. Acta*, **384**, 430–442.
- YAO, W., XIA, G., FANG, D. & JIANG, M. (1987). Studies on the calcium antagonistic action of tetrandrine: influence of tetrandrine and verapamil on hemodynamic action of ouabain in guinea-pig. *J. Tongji Med. Univ.*, **7**, 80–83.

(Received October 2, 1992

Revised January 23, 1993

Accepted February 10, 1993)