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Inhibitory effect of noradrenaline uptake inhibitors on contractions of rat aortic smooth muscle

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1 The effects of noradrenaline (NA) uptake inhibitors on contractions induced by NA, high K^+, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in rat isolated aorta were investigated.

2 Protriptyline (0.3 μM) and amitriptyline (0.3 μM) produced an approximately parallel shift to the right in the dose-response curve to NA. Protriptyline (>0.3 μM), amitriptyline (>0.3 μM) and xylamine (0.01–1 μM) significantly reduced the maximal contractile response to NA. The IC_{50} values for inhibition of the contractile response to 3 μM NA were 1.58 μM for xylamine, 1.70 μM for amitriptyline and 2.57 μM for protriptyline.

3 Protriptyline and amitriptyline dose-dependently inhibited the high K^+ (60 mM)-induced contraction (IC_{50} = 0.69 μM for protriptyline and IC_{50} = 3.15 μM for amitriptyline). In contrast, xylamine did not affect the high K^+-induced contraction.

4 Protriptyline and amitriptyline dose-dependently inhibited TPA (1 μM)-induced contraction in calcium-free solution; xylamine (up to 30 μM) was without effect. Staurosporine (10 nM) completely inhibited the TPA- and NA-induced contraction.

5 Protriptyline (3 μM) and amitriptyline (3 μM) caused about 54% and 60% inhibition, respectively, of aortic contractions caused by endothelin-1 (10 nM) in the absence of endothelium. Xylamine (10 μM) was without effect.

6 Inhibitory effects of NA uptake inhibitors on contractions were independent of the presence of endothelium and were unaffected by the K^+ channel blockers, tetraethylammonium ions (up to 3 mM) and glibenclamide (up to 30 μM).

7 These results indicate that tricyclic antidepressant drugs such as protriptyline and amitriptyline could act as both postsynaptic adrenoceptor antagonists and direct inhibitors of muscle contraction; whereas, xylamine, a structurally distinct NA uptake blocker might principally exert its action only at α-adrenoceptors on rat aortic smooth muscle.

Keywords: NA uptake inhibitor; protriptyline; amitriptyline; xylamine; 12-*O*-tetradecanoylphorbol-13-acetate; endothelin I; smooth muscle; endothelium; rat aorta

Introduction

The therapeutic action of tricyclic antidepressants in depression is mediated by inhibition of neuronal uptake of noradrenaline (NA) and/or other monoamines in the central nervous system. The undesirable effects are largely attributed receptors to their antagonistic effects at muscarinic and α-adrenoceptors in peripheral tissues (Klerman & Cole, 1965; Hrdina & Ling, 1970; Doggrell & Vincent, 1981; Rehavi *et al.*, 1987). For example, an inhibitory effect on vascular smooth muscle might be a cause of the hypotension common in patients undergoing tricyclic antidepressant therapy (Klerman & Cole, 1965). On the other hand, tricyclic agents at low concentrations were found to enhance the vascular responses to both electrical field stimulation and exogenous NA application (Kaumann *et al.*, 1965). The facilitatory effect is probably due to inhibition of NA uptake into the noradrenergic nerve terminals in the periphery and this increases the concentration of NA at the postsynaptic surface (Axelrod *et al.*, 1961; Iversen, 1965). Interference with calcium influx has also been proposed for the inhibitory effect on smooth muscle contraction of tricyclic antidepressants such as desipramine (Hrdina & Garattini, 1967; Hrdina & Ling, 1970). However, the nature of the vasorelaxant action of NA uptake inhibitors is still unclear.

Activation of α_1-adrenoceptors in vascular smooth muscle by NA is known to generate two second messengers; inositol 1,4,5-trisphosphate mobilizes the internal Ca^{2+} stores and diacylglycerol, a protein kinase C (PKC) activator might enhance Ca^{2+} influx through phosphorylation of Ca^{2+} channels (Villalobos-Molina *et al.*, 1982; Rapoport, 1987; Shearman *et al.*, 1989). Therefore, it is possible that NA uptake inhibitors interfere with any step along the NA-elicited signalling pathway to cause relaxation of arterial smooth muscle. The present study was undertaken to investigate the possible mechanisms underlying the inhibitory effect of these drugs on contraction in rat isolated aortic rings with different pharmacological manipulations. It was found that protriptyline and amitriptyline, two tricyclic antidepressants, and xylamine, a selective irreversible non-tricyclic blocker of NA uptake (Cho *et al.*, 1980; Ransom *et al.*, 1985) relaxed the arteries through distinct mechanisms. The former two agents appeared to act at multiple sites on arterial smooth muscle to cause vasorelaxation, e.g. non-competitive antagonism of α-adrenoceptors, inhibition of Ca^{2+} entry and interaction with excitation-contraction coupling. The latter agent seemed to relax arteries mainly through its antagonistic effect at α-adrenoceptors on smooth muscle in a non-competitive manner.

Methods

Preparations

Male Sprague-Dawley rats (~400 g) were killed by cervical dislocation and bled. Aortic rings of ~4 mm in length were prepared and mounted in 20-ml organ baths containing the

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Krebs-Henseleit (K-H) solution of the following composition (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 25, KH_2PO_4 1.2, D-glucose 11.1, ascorbic acid 0.2. The bath solution was maintained at 37±1°C and constantly oxygenated with 95% O_2+5% CO_2. The preparations were allowed to equilibrate for at least 1.5 h under 1 g resting tension. The isometric contraction was measured with force-displacement transducers (Grass Instrument Co.). In experiments using high K^+ solution, the equimolar amount of Na^+ was replaced by K^+ to maintain a constant ion strength.

Experimental protocols

Twenty minutes after setting up the organ baths, tissues were first contracted with a single dose of noradrenaline (NA, 3 μM) to test for their contractile responses after which they were rinsed three times in K-H solution to restore tension to the preconstricted level. The aortic rings were then contracted with NA or external K^+ applied cumulatively (ranging from 1 nM to 10 μM for NA and from 5 to 80 mM for external K^+) to obtain concentration-response curves. Once the maximal response to NA or external K^+ had been reached, preparations were washed with K-H solution every 20 min until the tension returned to the basal level. Tissues were then incubated with different concentrations of NA uptake blockers for 30 min and another cumulative concentration-response curve to NA or external K^+ was repeated.

In another series of experiments, sustained contractions of aorta to 3 μM NA, 60 mM external K^+ or 1 μM TPA were obtained and the NA uptake blockers were then applied cumulatively to induce inhibition. The effect of the vehicle was also tested. Experiments were also performed with Ca^{2+}-free K-H solution to which 0.5 mM EDTA had been added.

In some experiments the endothelial layer was removed mechanically by rubbing the lumen of the artery with plastic tubing. Successful removal of the endothelium was verified by the inability of the preparation to relax to 10 M acetylcholine at the start of each experiment.

Drugs

The following compounds were used: noradrenaline bitartrate, glibenclamide, tetraethylammonium chloride, prazosin hydrochloride, endothelin-1, 12-*O*-tetradecanoylphorbol-13-acetate (Sigma, ST. Louis, MO, U.S.A.), protriptyline, amitriptyline, xylamine and staurosporine (Research Biochemicals, Natick, MA, U.S.A.). Drugs were dissolved in calcium-free K-H solution except for prazosin, glibenclamide, TPA and straurosporine which were dissolved in dimethyl sulphoxide (DMSO); 0.2% DMSO in organ baths did not affect muscle contraction induced by agonists.

Statistics

To study the effect of NA uptake blockers on the agonist-induced contraction, values of EC_{50} and maximal tension were compared in the absence and presence of uptake blockers. The effects of the drugs on the sustained contraction were expressed as a percentage of the control value. Cumulative concentration-inhibition relationships were analysed with a non-linear curve fitting by a logistic equation (Grafit, Erithacus Software Limited) and IC_{50} was calculated as the drug concentration causing a half-maximal inhibition. Data were presented as arithmetic mean±s.e.mean of n experiments. A probability level of less than 0.05 was considered statistically significant.

Results

Effect of NA uptake blockers on the NA-induced contraction

NA contracted the rat aorta preparations with an EC_{50} of 0.12±0.01 μM and a maximal increase in tension of

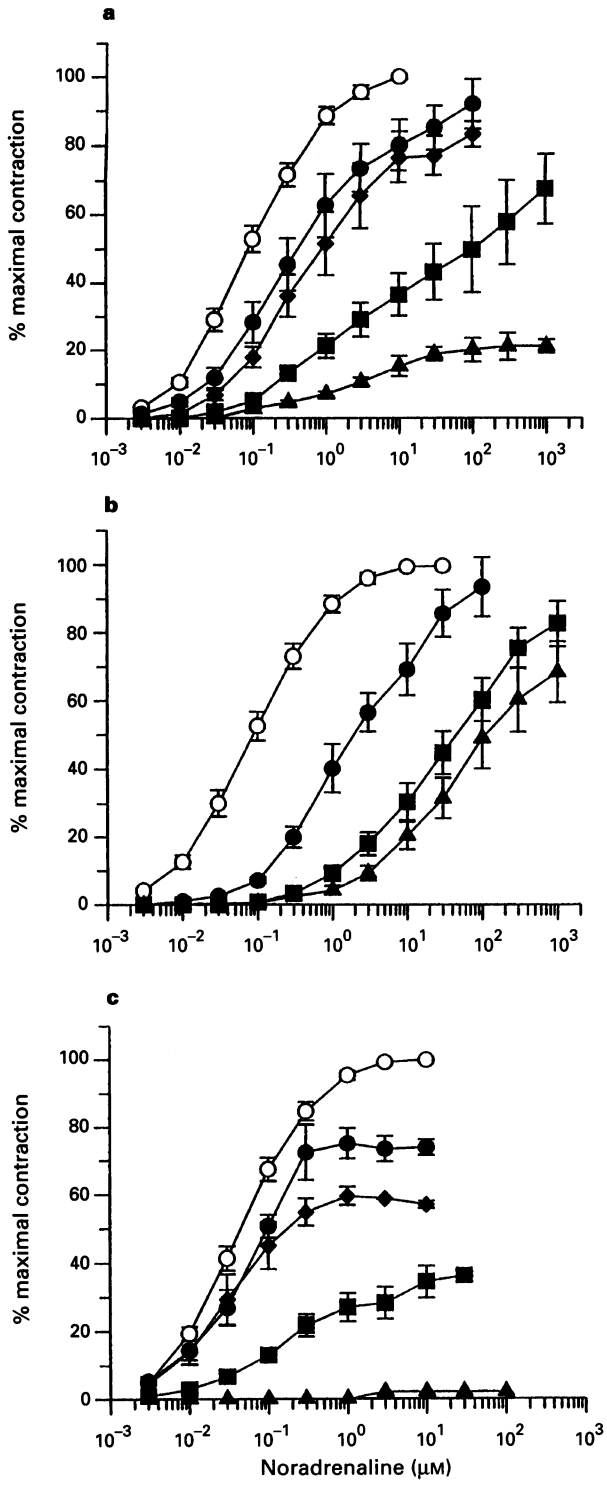

Figure 1 Logarithmic concentration-response curves of contractile responses of rat aorta for NA in the presence of NA uptake blockers. (a) Concentration-response curve for NA in the absence (○, control n=23) and presence of protriptyline (●, 0.3 μM n=7; ◆, 1 μM n=7; ■, 3 μM n=6; ▲, 10 μM n=3). (b) Concentration-response curve for NA in the absence (○, control n=23) and presence of amitriptyline (●, 0.3 μM n=7; ■, 3 μM n=8; ▲, 10 μM n=8). (c) Concentration-response curve for NA in the absence (○, control n=20) and presence of xylamine (●, 0.01 μM n=5; ◆, 0.03 μM n=5; ■, 0.1 μM n=7; ▲, 1 μM n=3). Drug was incubated for 30 min before repeating the second concentration-response curve. Data are expressed as a percentage of the maximal response obtained in the first (control) concentration-response curve. Curves are drawn by connecting the adjacent points. Values are mean±s.e.mean from n experiments.

0.99±0.03 g (n=66). None of three NA uptake blockers (protriptyline, amitriptyline and xylamine) affected the basal tension of the aortic muscle at concentrations of less than 10 μM. Following construction of a control curve for NA, the aortic rings were incubated for 30 min with different concentrations of each drug and a concentration-response curve for NA was again obtained. Low concentrations of protriptyline (0.3 μM, Figure 1a) and amitriptyline (0.3 μM, Figure 1b) caused an approximately parallel shift of the NA log concentration-response curve to the right. At higher concentrations protriptyline (>0.3 μM, Figure 1a), amitriptyline (>0.3 μM, Figure 1b) and xylamine (0.01–1 μM, Figure 1c) exerted an insurmountable inhibition, decreasing the magnitude of the maximal contraction and the slopes of the concenration-response curves for NA. Xylamine at concentrations greater than 1 μM inhibited almost completely the contractile response to NA.

In another set of experiments, submaximal steady contractions were induced by 3 μM NA and cumulative doses of the three inhibitors were added. The experimental traces in Figure 2 show that all three blockers inhibited the NA-induced contraction in a concentration-dependent manner with similar potencies (Figure 2d). IC_{50} values were 2.57 μM for protriptyline, 1.70 μM for amitriptyline and 1.72 μM for xylamine (n=7 for each drug, Table 1). The effects of protriptyline and amitriptyline were partially reversible, while the effect of xylamine was irreversible on repeated washout of the organ bath over 45 min (data not shown).

Effect of NA uptake blockers on the high K^+-induced contraction

To study the possible inhibitory actions of NA uptake blockers on Ca^{2+} influx, their effects on contractions induced by various concentrations of external K^+ were examined. The magnitude of tension development corresponds with graded increase in K^+ (5 to 80 mM) in the bath solution. When external K^+ was raised, it caused a concentration-dependent contraction in rat aorta with the maximal response at 60–80 mM. The high K^+-induced contraction was unaffected by prazosin (1 μM) indicating that noradrenergic nerve endings were not involved in the responses. Figure 3 shows that protriptyline (a) and amitriptyline (b) inhibited the high K^+-induced contraction in a non-competitive manner but the former was more potent. Both agents reduced the maximal response induced by high K^+ and the slope of the concentration-response curves (Table 1). Protriptyline and amitriptyline dose-dependently inhibited the high K^+-induced sustained contraction (Figure 4a and b) in a similar way to their effect on the NA-induced response. IC_{50} values were 0.69±0.03 μM for protriptyline and 3.15±0.29 μM for amitriptyline (Table 1, Figure 4d). However, xylamine did not affect the high K^+-induced contraction at concentrations causing a full inhibition of the NA-induced contraction in the same preparations (Figure 4c).

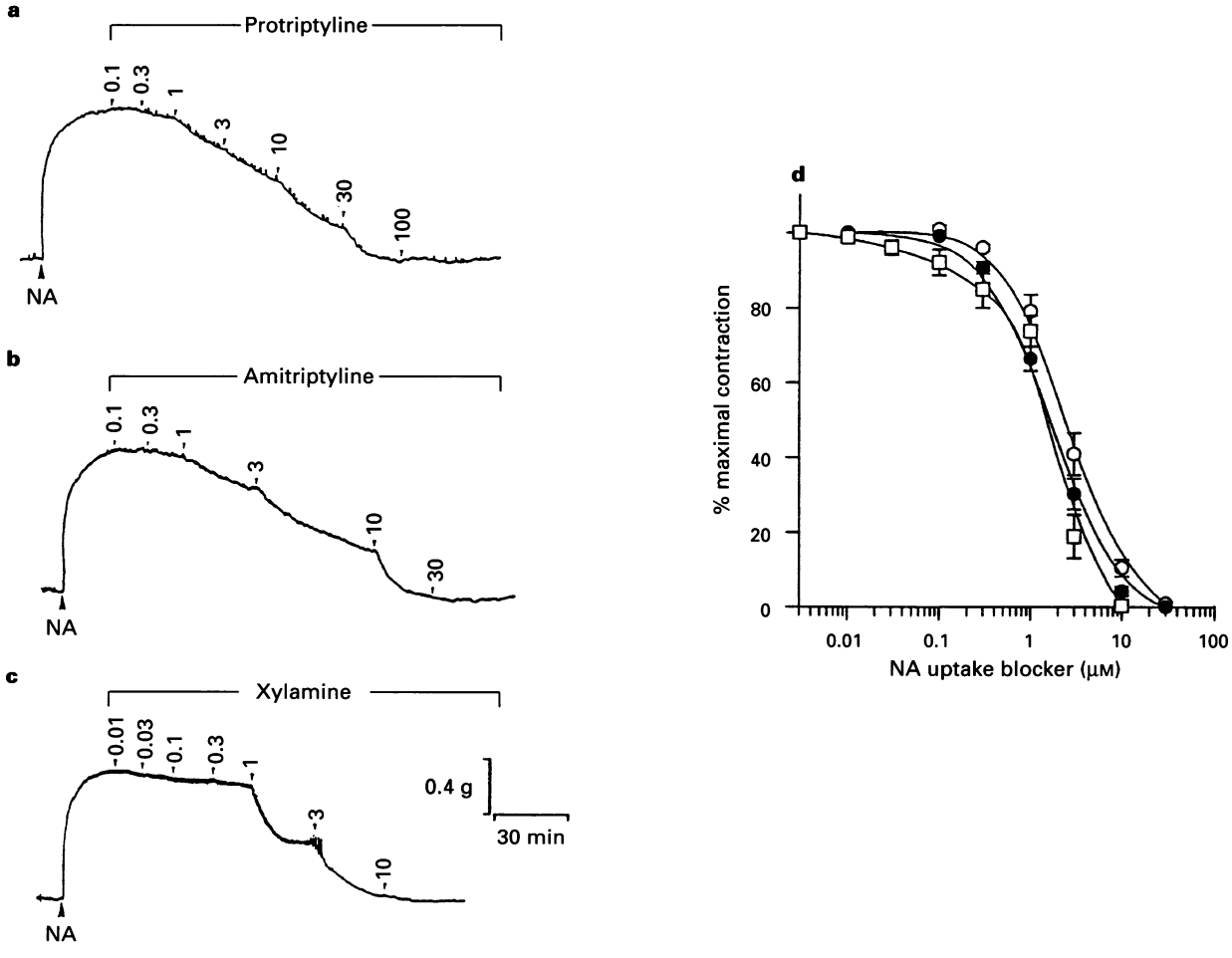

Figure 2 Traces are representative records for the relaxant effects of protriptyline (a), amitriptyline (b) and xylamine (c) on the NA-induced contraction in the rat isolated aorta. Protriptyline (○, n=6); amitriptyline (●, n=6) and xylamine (□, n=6) dose-dependently relaxed the NA (3 μM)-induced contraction. (d) Curves were drawn by fitting the data points to the logistic equation and gave respective IC_{50} values for the three inhibitors. Values are mean±s.e.mean from n experiments.

Table 1 Inhibitory effect of NA uptake inhibitors on contractions induced by NA and high K^+

Drugs	NA contraction IC_{50} (μM)	NA contraction Slope	n	K contraction IC_{50} (μM)	K contraction Slope	n
Protriptyline	2.57±0.32	1.64±0.23	7	0.69±0.03	2.67±0.26	6
Amitriptyline	1.70±0.09	1.75±0.14	7	3.15±0.29	2.13±0.16	6
Xylamine	1.72±0.24	2.40±0.46	7	n.a.	n.a.	

Values are means±s.e.mean of n experiments indicated. Tension development in response to 3 μM NA or 60 mM K^+ is expressed as 100% and percentage inhibition of the maximal tension by cumulative application of NA uptake blockers was calculated. Data points were fitted by a logistic equation to obtain values for IC_{50} and slope factor.

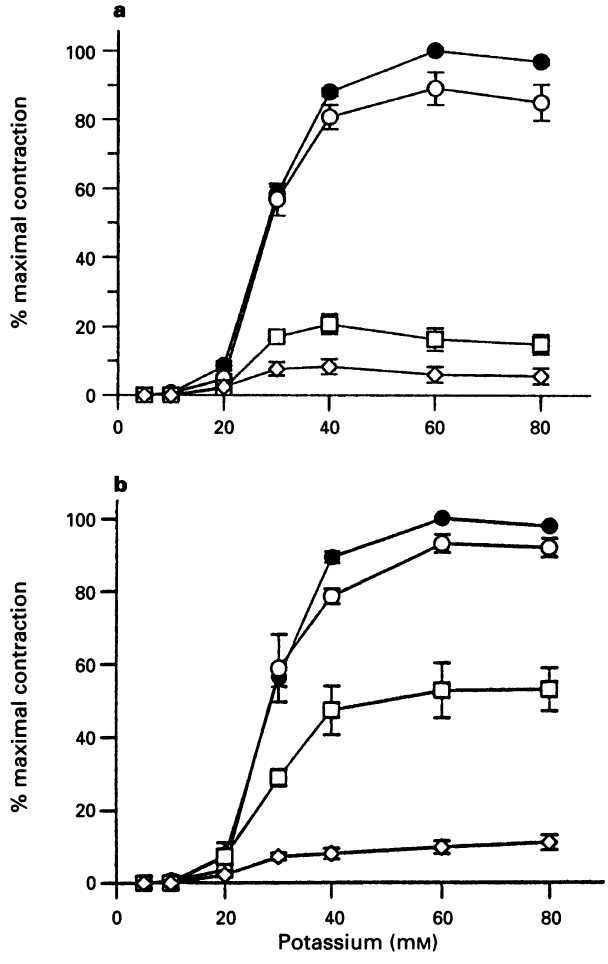

Figure 3 Effects of NA uptake blockers on the high K^+-induced contractions in rat aorta. (a) Concentration-response curves for KCl in the absence (●, $n=15$) and presence of protriptyline (○, 0.1 μM $n=5$; □, 1 μM $n=5$; ◇, 3 μM $n=5$). (b) Concentration-response curves for KCl in absence (●, $n=15$) and presence of amitriptyline (○, 0.3 μM $n=5$; □, 3 μM $n=5$; ◇, 10 μM $n=5$). Drug was incubated for 20 min before repeating the second concentration-response curve. All responses are expressed as a percentage of the maximal contraction at 80 mM K^+ in the absence of drugs. Values are mean±s.e. of mean from n experiments.

Effect of NA uptake inhibitors on the TPA-induced contraction

In order to test the possibility that NA uptake inhibitors also relax the NA-preconstricted aorta probably through Ca^{2+}-independent pathways, TPA, a protein kinase activator, was used to elicit contractions in the absence of extracellular Ca^{2+} (zero Ca^{2+} plus 0.5 mM EDTA). As shown in Figure 5a, TPA at 1 μM produced a slowly-developing contraction and the maximal sustained tension was 1±0.05 g ($n=20$). Similar results have been reported for 12-deoxyphorbol 13-isobutyrate, another PKC activator, which elicited a sustained contraction with a slow rate of rise without causing detectable changes in $[Ca^{2+}]_i$ in Ca^{2+}-free medium in rat aortic stripes (Nakajima *et al.*, 1991). The cumulative application of protriptyline and amitriptyline at concentrations above 0.1 μM caused relaxation of the rat aorta preconstricted by TPA with IC_{50} values of 7.1±2.9 μM ($n=7$) and 16.6±6.7 μM ($n=5$) respectively. In contrast, xylamine at concentrations up to 30 μM did not affect the TPA-induced tension ($n=6$). Figure 5b summarizes the concentration-dependent relaxation of the TPA-induced contraction by protriptyline and amitriptyline. In addition, staurosporine, a protein kinase C inhibitor, at 10 nM completely reverse contractions induced by TPA in Ca^{2+}-free solution and by NA in normal K-H solution ($n=4$, data not shown).

Effect of NA uptake inhibitors on the endothelin-1-induced contraction

Since protriptyline and amitriptyline inhibited the TPA- and the high K^+-induced contractions at different calcium concentrations, it is possible that their effect on the NA-induced contractile response might not be specific at adrenoceptors on smooth muscle plasma membrane as indicated by other investigators (Klerman & Cole, 1965; Hrdina & Ling, 1970). Endothelin-1 was used to contract the aorta to test this hypothesis. Endothelin-1 dose-dependently increased muscle tension in rat aortic rings; however, its action was not sustained and the tissue became desensitized to endothelin-1 (data not shown). After removal of the endothelium, the endothelin-1-induced contraction remained constant for over 1.5 h at a concentration of 10 nM. Protriptyline (3 μM) and amitriptyline (3 μM) reduced the endothelin-1-induced contractions by 54±2.6% ($n=4$) and 59.5±9.7% ($n=4$), respectively; the contact time was 90 min; however, xylamine (up to 100 μM) was without effect.

Effect of NA uptake blockers in the presence and absence of endothelium

Removal of the endothelial layer did not alter the effect of the NA uptake blockers on the NA-evoked contraction in rat aorta. There was no significant difference in inhibition by NA uptake inhibitors of contractions in the absence and presence of endothelium (Table 2). This rules out the possible involvement of endothelium-derived relaxing or hyperpolarizing factors. In addition, blockers of K^+ channels such as tetraethylammonium (up to 3 mM) and glibenclamide (up to 30 μM) at concentrations much greater than their K_d for inhibition of Ca^{2+}-activated and ATP-sensitive K^+ channels in arterial smooth muscle, respectively (Standen *et al.*, 1989; Huang *et al.*, 1990), did not alter the relaxant effect of NA

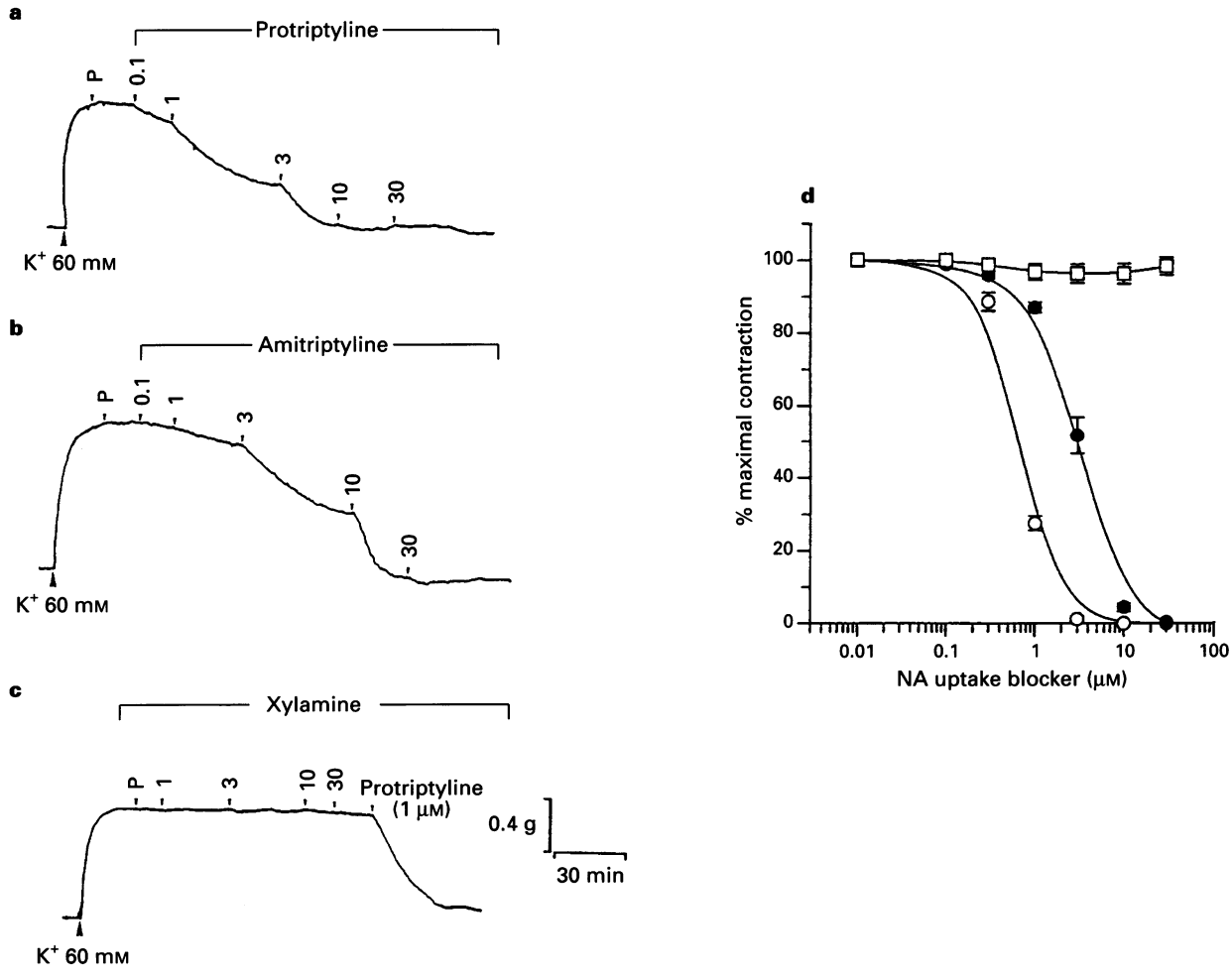

Figure 4 Traces are representative records for the effects of protriptyline (a) amitriptyline (b) and xylamine (c) on the high K^+-induced contraction in rat isolated aorta. Protriptyline and amitriptyline dose-dependently relaxed the high K^+ (60 mM)-induced contraction. The high K^+-induced contraction was unchanged by prazosin (P) at 1 μM. (d) Curves were drawn by fitting the data points to the logistic equation and gave respective IC_{50} values for protriptyline (○, $n=6$) and amitriptyline (●, $n=6$). Xylamine (□, $n=6$) did not affect the contractile response to high K^+. Values are mean ± s.e. of mean from n experiments.

uptake inhibitors, indicating that NA uptake inhibitors do not affect K^+ conductance in aortic smooth muscle.

Discussion

In this study, the effects of NA uptake inhibitors, protriptyline, amitriptyline and xylamine on the tension development in response to NA, high K^+, TPA and endothelin-1 were investigated in the rat isolated aorta. The results show that the three NA uptake blockers relaxed the rat aorta preconstricted by NA in a concentration-dependent fashion. It is clear that the relaxant effects of these agents were independent of the presence of endothelium. The lack of effect of TEA^+ and glibenclamide rules out the involvement of Ca^{2+}-activated or ATP-sensitive K^+ channel activation in the vasorelaxation induced by NA uptake inhibitors.

Protriptyline and amitriptyline at lower concentrations tested, showed a competitive antagonism against the NA-induced contractile response in the rat aorta preparations, whilst, xylamine was a non-competitive antagonist. At higher concentrations all three NA uptake blockers decreased the maximal contraction and the slope of the concentration-contraction curve, suggesting that NA uptake inhibitors are non-competitive antagonists of α-adrenoceptors in aortic smooth muscle. These results accord with the non-competitive inhibition of the NA-induced increase by desipramine in perfusion pressure of rat renal artery (Hrdina & Ling, 1970). Apparently, tricyclic antidepressants work differently from selective α_1-adrenoceptor antagonists, such as prazosin and benoxathian, in inhibiting the NA-induced contractile response. The latter two agents caused a parallel shift of the concentration-response curve to the right even at high concentrations without reducing the magnitude of the maximal response in the same preparation (Aboud *et al.*, 1993). Xylamine, a β-halobenzylamine compound previously found to block NA uptake into both central and peripheral adrenergic nerve terminals (Cho *et al.*, 1980; Ransom *et al.*, 1985) is structurally distinct from tricyclic antidepressants. Interestingly, this agent also inhibited the NA-induced contraction. All three agents reduced the maximal response to NA; this raises a possibility that they might act at sites other than α-adrenoceptors in smooth muscle. Indeed, protriptyline and amitriptyline decreased the maximal contraction to high K^+ and the slope of the concentration-contraction curve, as well as dose-dependently inhibiting the high K^+-induced sustained contraction. IC_{50} values are in the same range as those for the NA-induced response. Xylamine, by contrast, did not affect contractions evoked by high K^+. High K^+ has been demonstrated to evoke smooth muscle contraction by promoting Ca^{2+} entry through voltage-dependent Ca^{2+} channels which are readily activated by membrane depolarization (van Breemen & McNaughton, 1970). Therefore, extracellular Ca^{2+} entry is thought to be the major cause of high K^+-induced contractions. These findings

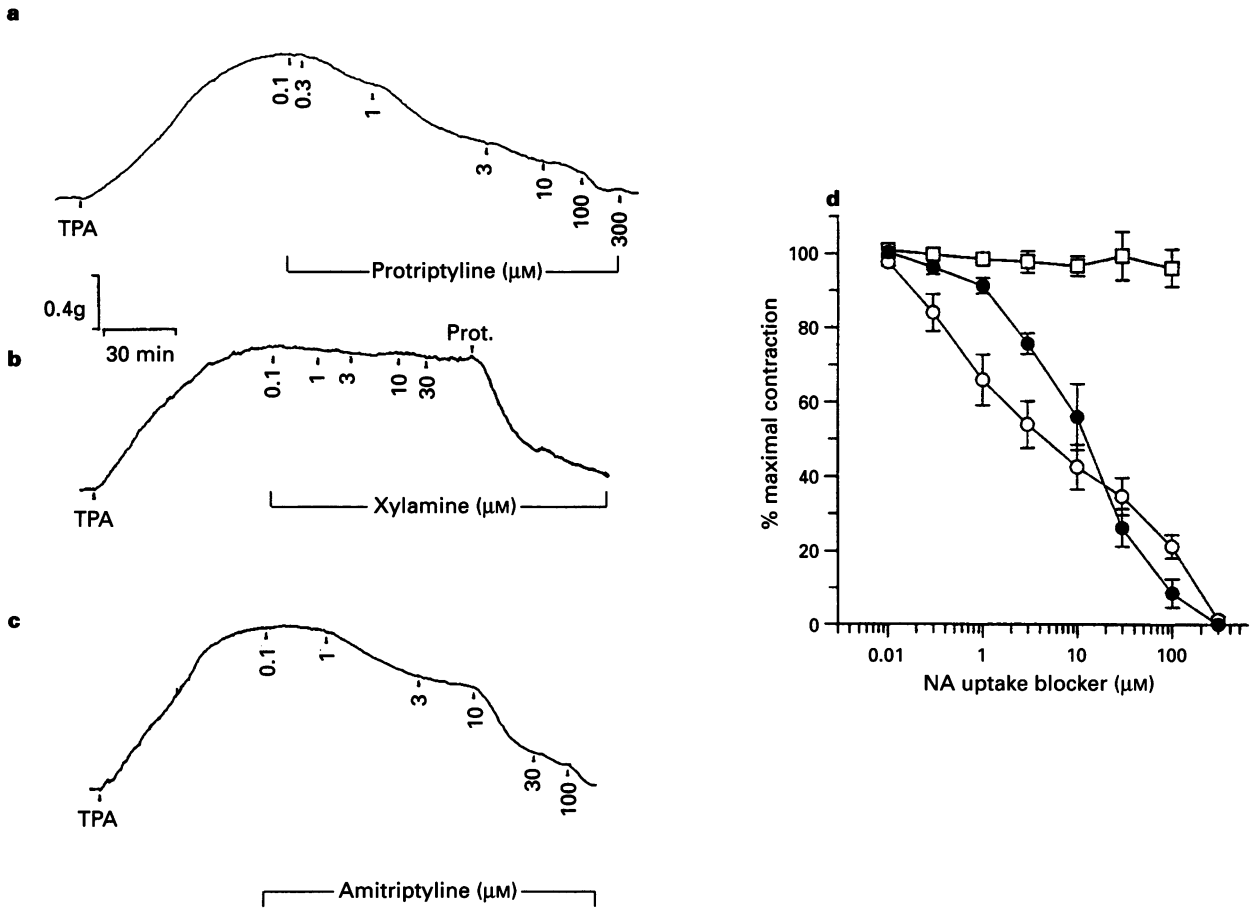

Figure 5 Traces are representative records for the relaxant effects of NA uptake blockers on the TPA-induced tension in aortic rings. Protriptyline (a) and amitriptyline (c) dose-dependently inhibited sustained contractions caused by 1 μM TPA in K-H solution containing 0.5 mM EDTA but no calcium ions. Xylamine (b) at concentrations up to 30 μM did not change the TPA-induced contraction. (d) Concentration-dependent relaxation curves for protriptyline (○, $n=7$), amitriptyline (● $n=7$) and xylamine (□, $n=6$). Curves were drawn by connecting the adjacent points and values are mean ± s.e. of mean from n experiments.

Table 2 Effect of endothelium on action of NA uptake inhibitors in aorta

Drugs (3 μM)	*% inhibition of NA contraction* *With endothelium*	n	*Without endothelium*	n
Proptriptyline	59.1 ± 5.6	6	62.1 ± 7.3	6
Amitriptyline	69.8 ± 4.2	6	59.1 ± 3.9	6
Xylamine	90 ± 5.2	7	93.6 ± 4.5	7

Values are means ± s.e.mean of n experiments indicated. A single dose of NA uptake inhibitors (3 μM) was used to inhibit contraction induced by 3 μM NA in the presence and absence of endothelium. Inhibitory effects of drugs are expressed as percentage inhibition of the NA-induced contraction.

indicate that tricyclic antidepressants could inhibit Ca^{2+} entry through the depolarized cell membrane in rat aorta and thus support the early report of antagonism between Ca^{2+} and desipramine in the rat perfused renal artery (Hidina & Garattini, 1967). Lack of effect of xylamine on the depolarization-induced contraction suggests that it might act only at α-adrenoceptors without interfering with Ca^{2+} influx.

It is generally believed that the NA-induced sustained tension is mediated by elevated cytoplasmic Ca^{2+} levels and PKC is involved in promoting Ca^{2+} influx through Ca^{2+} channels (Shearman *et al.*, 1989). Both inorganic Ca^{2+} channel blockers and PKC inhibitors inhibit tonic contraction in response to NA in rat aorta (Shimamoto *et al.*, 1993; personal observations). In addition, the sustained contraction occurs without measurable sustained increase of $[Ca^{2+}]_i$ (Aksoy *et al.*, 1983). Thus, PKC activators induce slowly developing tone in vascular smooth muscle, which is not associated with phosphorylation of Ca^{2+}-calmodulin-dependent phosphorylation of myosin light chain but related to phosphorylation of intermediate filaments (Rasmussen *et al.*, 1984; 1987). The present study shows that TPA produced a slow tonic contraction in the absence of extracellular Ca^{2+}, indicating that PKC activation can interact with contractile filaments at the resting $[Ca^{2+}]_i$. Protriptyline and amitriptyline reduced the TPA-induced ten-

sion in a dose-dependent manner. Xylamine, however, did not have any inhibitory effect. In comparison to their effect on the high K^+-induced contraction, a slightly higher concentration of both agents was needed to cause half-maximal inhibition of the TPA-induced response. These results suggest that the PKC-mediated steps in excitation-contraction coupling may also be the site of action for tricyclic antidepressant agents when they are used at higher concentration. In addition, protriptyline, amitryptyline and PKC inhibitors reduced contraction to endothelin-1 which has been shown to activate the same intracellular second messenger pathways as found for NA in vascular smooth muscle (Resink *et al.*, 1988). However, xylamine, a structurally different blocker of NA uptake inhibited only the NA-induced contraction without affecting responses to high K^+, TPA or endothelin-1. These results clearly indicate that xylamine may act only as a non-competitive antagonist of α-adrenoceptors on aortic smooth muscle.

In summary, the present results demonstrate that at low concentrations, both protriptyline and amitriptyline non-selectively antagonized the contractile effect of NA on the rat aorta. Inhibition of Ca^{2+} entry through the cell membrane may contribute to the antagonism, and at higher concentrations inhibition of the PKC-mediated contractile mechanism may be involved. Xylamine, on the other hand, appeared to act solely on α-adrenoceptors as a non-competitive antagonist in the rat isolated aorta.

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