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Effect of Nolinium Bromide on Vascular Smooth Muscle (41897)

ROBERT R. BROOKS, SANDRA M. JONES, AND ALAN F. MOORE

Research Division, Norwich Eaton Pharmaceuticals, Inc., Norwich, New York 13815-0191

Abstract. Nolinium bromide inhibits gastric acid secretion and gastrointestinal smooth muscle contraction. While the compound's gastric antisecretory action has been attributed in part to inhibition of gastric adenylate cyclase and the gastric proton-transport ATPase, the mechanism of nolinium bromide's relaxant effect on smooth muscle has not been elucidated. We have determined that nolinium bromide inhibits contraction of vascular smooth muscle, using isolated rabbit aortic strips. This report characterizes the specificity of this inhibition (by using several agonists) and its Ca^{2+} dependence (by using contractile conditions of known dependence on various Ca^{2+} pools). Nolinium bromide (50–200 μM) was a reversible, insurmountable inhibitor of contractions induced by Ca^{2+} (in 40 mM KCl depolarizing medium) and norepinephrine, with IC_{50} values of 96 and 118 μM, respectively, for suppression of maximum contractile force. Contractions in response to Asn^{1}Val^5-angiotensin II in both 2.5 mM Ca^{2+} and 0 mM Ca^{2+} medium were also inhibited (IC_{50} value = 110 μM under both conditions). Initial contraction rates for all these conditions were depressed by nolinium bromide. Nolinium bromide inhibited equally the phasic (internal Ca^{2+}-dependent) and tonic (external Ca^{2+}-dependent) components of norepinephrine-induced contractions. These results extend the muscle relaxant profile of nolinium bromide to include, albeit with low potency, vascular smooth muscle, and show that its potency is similar in inhibiting the contractile response to three different stimuli: angiotensin II, Ca^{2+}, and norepinephrine.

Nolinium bromide (NB)[1] [2-(3,4-dichlorophenylamino)quinolizinium bromide] is a nonanticholinergic, gastric acid antisecretory agent and a gastrointestinal tract antispasmodic agent (1–3). The gastrointestinal antispasmodic action of NB has been demonstrated in a variety of test systems. The compound inhibited electrically induced contractions of rabbit ileum and nicotine-induced contractions of rat ileum *in vitro,* gastric emptying in fasted rats, and intestinal transport of a charcoal meal in mice. In the anesthetized dog, NB antagonized colonic contractions induced by acetylcholine, histamine, serotonin, and pelvic nerve stimulation, and duodenal contractions due to vagal stimulation and acetylcholine. In the unanesthetized dog, feeding-induced colonic and duodenal motilities were inhibited by NB (2, 3).

The antisecretory action of NB may involve inhibition of enzymes of gastric acid secretion, specifically histamine-stimulated adenylate cyclase (4) and potassium-stimulated ATPase (5). NB has no direct histamine-H_2 receptor blocking properties, since it did not inhibit histamine-stimulated acid secretion from isolated guinea pig gastric discs (5). In contrast, the antispasmodic action of NB has not been explored mechanistically. Nor has its effect on vascular smooth muscle been tested *in vitro.*

In this study we determined the effects of NB on contractions of isolated rabbit aortic strips, a tissue not previously studied with this agent. The experiments reported here were designed to determine if NB had an effect on vascular smooth muscle *in vitro,* whether there was specificity in that effect with respect to which agonists are sensitive to NB, and whether NB's action could be connected to an effect on Ca^{2+} supply. A preliminary report of these results has been presented (6).

Materials and Methods. *Chemicals and solutions.* Normal Krebs–Ringer solution contained 1.16 mM $MgSO_4$, 4.69 mM KCl, 1.30 mM NaH_2PO_4, 2.48 mM $CaCl_2$, 5.55 mM dextrose, 118 mM NaCl, and 25 mM $NaHCO_3$. Calcium-free Krebs was of the same composition but without the $CaCl_2$. High-potassium depolarizing Krebs lacked $CaCl_2$ and contained 40 mM KCl. These solutions were

[1] Abbreviations used: ANG II, Asn^{1}Val^5-angiotensin II; NB, nolinium bromide.

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prepared fresh daily. The pH of these solutions was 7.4 upon equilibration by aeration with 95% O_2–5% CO_2 at 37°C.

Nolinium bromide and angiotensin II (ANG II = Asn–Arg–Val–Tyr–Val–His–Pro–Phe) were synthesized at Norwich Eaton Pharmaceuticals, Inc. NB (mol mass 370.1 g/mole) was dissolved fresh daily in the medium appropriate to the individual experiment. ANG II was prepared as a 1 m*M* stock solution in distilled water containing 19 mg mannitol/ml and was stored frozen. $CaCl_2$ was kept refrigerated as a 0.5 *M* stock solution in distilled water. Norepinephrine (L-arterenol bitartrate, Sigma) was prepared fresh daily as a 3.16 m*M* stock solution in 0.1 *N* HCl.

Animals. Female New Zealand White rabbits, 2.5–3.5 kg, were obtained from Dutchland Breeding Laboratories (Denver, Pa.) and Sunrise Laboratory Animals (Whitehouse Station, N.J.). Rabbits were housed in metal cages under standard laboratory conditions with a 12-hr dark/light cycle.

Tissue preparation. As described by Furchgott and Bhadrakom (7), a rabbit was killed by cervical dislocation and the thoracic aorta was rapidly excised and placed in a petri dish containing oxygenated Krebs solution at room temperature. Connective tissue was carefully removed and the muscle was cut helically into a single strip (approximately 0.25 × 8 cm) and then was cut transversely into four equal segments. Each strip was attached at one end to a glass tissue holder with surgical silk (No. 3-O) and placed into a 25-ml tissue bath containing the appropriate medium at 37°C. The opposite end of the strip was attached to a Grass FT-03C force-displacement transducer coupled to a Grass Model 7B polygraph (Grass Instrument Co., Quincy, Mass.). Baths were aerated with 95% O_2–5% CO_2 and the bath medium was changed every 20 min during the relaxation period. This period generally lasted 2 hr with the strip under 2 g of tension.

Experimental procedure. In all experiments a cumulative concentration–response curve for ANG II was obtained by cumulative additions of aliquots of ANG II to give final bath concentrations of 1 to 128 n*M*. The bath medium was changed at 15–20 min intervals for 1–2 hr until a stable baseline was again observed. The control concentration–response curve was then determined using the desired agonist (1–128 n*M* ANG II, 1–128 n*M* ANG II in calcium-free medium, 0.0125–20 m*M* Ca^{2+} in calcium-free depolarizing medium, or 0.01–32 μM norepinephrine). The medium was changed as above and, after a stable relaxed condition was again achieved, the bath medium was replaced with medium containing the desired concentration of NB, and within 5 min a cumulative concentration–response curve was started for the agonist. One of the four strips was not exposed to NB and was carried through the experiment as a control for time-related changes in strip behavior. In most experiments a recovery control curve was determined at the end of the experiment after NB was removed and a stable baseline obtained.

Data analysis. Each point (g tension) of the control and experimental concentration–response curves was expressed as a percentage of the maximum control curve contraction for that tissue. Corrections for time-related changes in tissue responsiveness were made if the time control strip not exposed to NB showed a change in maximum contraction greater than or equal to 10% of the first control curve for that strip. In this event, the correction was achieved by multiplying each experimental curve point (g tension) by the ratio of the maximum tensions elicited in the time-control strip in control and test periods. For example, if the time control strip gave maximum tensions of 5 and 4.4 g in the first and second curves, respectively, then a time correction would be made by multiplying all experimental strip second curve points by 5/4.4.

Plots of log agonist concentration versus percentage of maximum contraction were made for control strips and a straight line was fitted to the appropriate portion by the method of least squares. The EC_{50} value; that is the agonist concentration causing 50% of the maximum uninhibited contraction, was calculated from the equation of the line. The IC_{50} value, i.e., the concentration of NB which inhibited maximum contractile force 50%, was calculated analogously for each agonist. Initial rates of tension development were estimated by laying a straight edge on the polygraph record. For this purpose the portion of the concentration–response curve including one-half the maximum tension developed was used. The resulting initial rates of contraction

at one-half maximum tension were expressed in units of grams per minute. Rates were also expressed as a percentage of the individual tissue's control response to the agonist alone. Corrections for time-related changes were made as described above by referring to a concurrently run control strip's behavior.

Results. *Contractile effects of angiotensin II, calcium, and norepinephrine.* Rabbit aortic strips displayed concentration-dependent contractions to ANG II in normal medium. The maximum contraction varied somewhat from strip to strip from the same animal, but the between-animal variability was greater. The EC_{50} value, i.e., the agonist concentration causing 50% of the maximum contraction, for ANG II in normal Krebs medium was 5.80 ± 0.20 n*M* (mean ± SE, $N = 3$). In calcium-free medium, ANG II was three times less potent (EC_{50} value = 17.8 ± 1.2 n*M*, $N = 6$) and maximum contractile tension was reduced to 41% as compared to normal calcium conditions (Table I). Ackerly *et al.* (8) observed EC_{50} values of 13 and 60 n*M* for ANG II under normal and calcium-free conditions, respectively, and removal of calcium reduced maximum contractile force to 30% of control. Thus the present results with ANG II are comparable to those reported previously. Calcium was a considerably less potent agonist than ANG II, with an EC_{50} value of 77 ± 4 μM ($N = 10$). Norepinephrine was also less potent than ANG II (EC_{50} value = 68.3 ± 1.0 n*M*, $N = 3$).

Maximum contractile force varied with the agonist (Table I). The greatest maximum tension (3.28 g) was produced by Ca^{2+} in 40 m*M* KCl, followed by norepinephrine (2.88 g), ANG II in normal medium (2.20 g), and ANG II in the absence of added Ca^{2+} (0.91 g). The initial rate of force development also differed under the four stimulating conditions (Table

TABLE I. INHIBITION BY NOLINIUM BROMIDE OF CONTRACTION OF ISOLATED RABBIT AORTIC STRIPS

Agonist	Nolinium bromide concn (μM)	Number of tissues	Maximum contractile force (g) (mean ± SE)	Percentage of maximum control contraction (mean ± SE)	IC_{50} (μM) (95% limits)
Angiotensin II in 2.5 m*M* Ca^{2+}	0	12	2.20 ± 0.33	100	
	50	3	2.14 ± 0.07	88.5 ± 2.4	110
	100	6	1.40 ± 0.04	58.0 ± 1.3	(82–146)
	200	6	0.35 ± 0.02	16.5 ± 0.7	
Angiotensin II in Ca^{2+}-free buffer	0	8	0.91 ± 0.11	100	
	50	2	0.76 ± 0.01[a]	90.7 ± 9.3	110
	100	2	0.57 ± 0.33[a]	57.2 ± 10.0	(39–344)
	200	2	0.20 ± 0.20[a]	17.1 ± 17.1	
$CaCl_2$ in 40 m*M* KCl	0	12	3.28 ± 0.17	100	
	50	4	2.56 ± 0.27	80.9 ± 3.2	96
	100	4	2.19 ± 0.36	57.3 ± 6.8	(58–158)
	200	4	0.20 ± 0.11	5.4 ± 2.7	
Norepinephrine	0	4	2.88 ± 0.10	100	
	50	3	2.43 ± 0.16[b]	84.5 ± 1.7	118
	100	3	1.76 ± 0.11[b]	61.2 ± 2.1	(90–155)
	200	3	0.73 ± 0.06[b]	25.4 ± 2.0	

Note. Segments of helically cut aortic strips were contracted by cumulatively increasing concentrations of the indicated agonist in the absence and presence of the indicated concentration of nolinium bromide (NB). The maximum agonist concentrations were 128 n*M*, 5 m*M*, and 31.6 μM for angiotensin II, $CaCl_2$, and norepinephrine, respectively. Percentage of maximum control contraction, based on the strip's response before NB, was calculated after tensions were corrected for time-related changes as described under Materials and Methods. The IC_{50} values (concentration giving 50% inhibition) for NB were estimated by least-squares regression analysis.

[a] Corrected for 33 and 36% time-related decrease in the maximum contractile force observed in control strips. Actual tensions in strips treated with NB were lower than shown.

[b] Corrected for 15, 19, and 22% time-related increase in the maximum contractile force observed in control strips. Actual tensions in strips treated with NB were higher than shown.

TABLE II. EFFECT OF NOLINIUM BROMIDE ON INITIAL RATE OF TENSION DEVELOPMENT OF RABBIT AORTIC STRIPS

Agonist	Nolinium bromide concn (μM)	Number of tissues	Initial contraction rate (mean ± SE) (g/min)	Percentage of control rate (mean ± SE)
Angiotensin II in 2.5 mM Ca^{2+}	0	4	0.264 ± 0.016	100
	50	3	0.226 ± 0.026	85.1 ± 2.6
	100	3	0.128 ± 0.009	48.8 ± 3.1
	200	3	0.043 ± 0.006	16.1 ± 0.9
Angiotensin II in Ca^{2+}-free buffer	0	4	0.108 ± 0.021	100
	50	2	0.138 ± 0.002	82.0 ± 1.2
	100	2	0.090 ± 0.030	107 ± 35
$CaCl_2$ in 40 mM KCl	0	8	0.160 ± 0.011	100
	50	4	0.084 ± 0.005	58.9 ± 7.3
	100	4	0.134 ± 0.019	79.0 ± 11.4
Norepinephrine	0	4	1.67 ± 0.240	100
	50	3	0.908 ± 0.127	53.7 ± 8.0
	100	3	0.591 ± 0.017	35.8 ± 6.5
	200	3	0.291 ± 0.055	16.5 ± 0.9

Note. Observed initial contraction rates for tissues exposed to nolinium bromide were corrected for time-related changes in a concurrently run control strip. Observed changes in the control strip initial rate were −2 to +36% for angiotensin in 2.5 mM Ca^{2+}, −46 to −52% for angiotensin in 0 mM Ca^{2+}, +42 to +86% for Ca^{2+} in 40 mM KCl, and +50 to +190% for norepinephrine.

II). Norepinephrine produced the largest initial rate (1.67 g/min). Compared with norepinephrine, the rate of tension development was five times lower (0.26 g/min) with ANG II in normal medium, and lower still with Ca^{2+} (0.16 g/min) and ANG II in calcium-free medium (0.11 g/min).

Tissues in medium lacking added Ca^{2+} showed a progressive decrease in both maximum tension development and initial contraction rate (Table II) in response to ANG II. This was due to the low Ca^{2+} conditions, since tissues in normal medium (2.5 mM Ca^{2+}) retained responsiveness to ANG II. With both norepinephrine and Ca^{2+} in depolarizing medium, maximum contractile force increased moderately (<25%) with time and on repeated exposure to the agonist. With these same stimulants, even more pronounced increases in the initial rate of tension development were observed on repeated exposure, i.e., increases of up to 86% for Ca^{2+} and 190% for norepinephrine (Table II).

Nolinium bromide effects. NB inhibited ANG II-induced contractions in normal medium (Fig. 1). Curves were shifted to the right and maximum tension was depressed as compared to control curves. Both effects were a function of inhibitor concentration. At 50, 100, and 200 μM NB, maximum tension was inhibited 12, 42, and 83%, respectively (Table I). If NB acts by inhibiting Ca^{2+} movement into the cell, it should be less effective in inhibiting ANG II-induced contractions of aortic strips in calcium-free medium, since such contractions depend on internal Ca^{2+} in stores (8). In fact, concentration-dependent inhibition was observed under calcium-free conditions. At 50, 100, and 200 μM NB maximum tension development was inhibited 9, 43, and 83%, respectively. For ANG II under both conditions (2.5 and 0 mM Ca^{2+}), the IC_{50} value of NB was estimated as 110 μM (Table I).

To determine if inhibition by NB was specific for ANG II, both Ca^{2+} and norepinephrine were used as agonists. NB also inhibited these contractions in a concentration-dependent fashion (Table I). The main difference between the effects of NB on these agonists compared with its effects on ANG II-induced contractions was the apparently increased sensitivity of calcium- and norepinephrine-induced contractions to the lowest concentration of NB. The 50 μM-inhibited curves

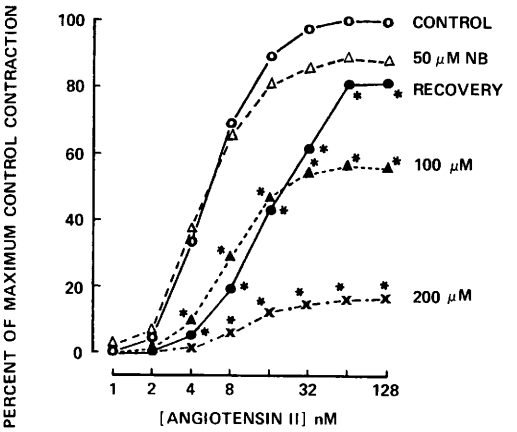

FIG. 1. Inhibition by nolinium bromide (NB) of contractions of rabbit aortic strips induced by angiotensin II (ANG II) in normal medium. Strips were suspended in Krebs–Ringer solution containing 2.5 m*M* $CaCl_2$ at 37°C under 2 g tension and contracted by cumulatively increasing concentrations of ANG II in the absence and presence of NB. Recovery curves were generated after removal of the inhibitor by several changes of the bath medium. Starred points are significantly different ($P < 0.01$) from the corresponding control contraction by a *t* test ($N = 3$). Maximum control contractions were 2.3–2.4 g.

were much more displaced to the right for Ca^{2+} (Fig. 2) and for norepinephrine (data not shown) than for ANG II (Figs. 1 and 2). However, maximum tension development was similarly inhibited with IC_{50} values estimated as 96 and 118 μM for calcium- and norepinephrine-induced contractions, respectively (Table I).

NB inhibited initial rates of tension development in a concentration-related manner for ANG II (normal medium) and for norepinephrine (Table II). Effects on this parameter paralleled effects on maximum tension (Table I). Concentration-independent effects on initial contraction rate were observed with ANG II in calcium-free medium and with Ca^{2+} in 40 m*M* KCl.

After strips were washed with medium lacking NB, they were able to respond again to the various agonists. Maximum post-NB contractions ranged from 46 to 112% of control values (Table III). As shown in Fig. 1, however, the recovery curve for ANG II in normal medium was displaced to higher agonist concentrations. Similar shifts to the right were observed with the other agonist conditions (data not shown). Only partial recovery (46%) was found with ANG II in calcium-free medium for strips exposed to 200 μM NB.

Discussion. This study was undertaken to determine if NB inhibited contraction of vascular smooth muscle *in vitro* and further to determine if such inhibition were specific with respect to agonist or could be related to effects on Ca^{2+} supply. Rabbit aortic strips were useful, since different components of the calcium-dependence of contraction in this tissue are identifiable by use of different agonists. Thus, the tonic component of norepinephrine-induced contractions has been ascribed to the movement of external Ca^{2+} into the cytoplasm (9, 10), while the rapid phasic component is due to internal Ca^{2+} stores (11, 12). Complete removal of external Ca^{2+} therefore only attenuates, but does not abolish, the ability of norepinephrine to contract the aorta. ANG II, by use of internal Ca^{2+} stores, is also able to contract the rabbit aortic strip in calcium-free medium (8, 13). Contractions induced by Ca^{2+} in potassium-depolarizing medium seem to be dependent entirely on extracellular Ca^{2+} entering the cell through potential-dependent channels operating when K^+ causes membrane depolarization (12, 14).

Although NB clearly inhibited aortic strip contractions, it did so without much evidence of specificity. The IC_{50} values for NB antagonism of ANG II, $CaCl_2$, and norepinephrine were all approximately equal (100 μM) and reflected low potency. Both the lack of agonist specificity and low potency *in vitro* make NB's effects on vascular smooth muscle analogous to its antispasmodic action on gastrointestinal smooth muscle. Goldenberg (2) reported pA_2 values of 5.3 and 4.3 for inhibition of acetylcholine- and histamine-induced contractions, respectively, of guinea pig ileum.

The role of transmembrane calcium flux and membrane depolarization in the contractile response of rabbit aortic strips to several agonists has been previously studied by one of the present authors (8). It was reported that ANG II-induced contractions were insensitive to 10 μM verapamil, but partially inhibited by another calcium entry blocker, SKF-525A, as well as by use of calcium-free buffer. These results suggested that ANG II-

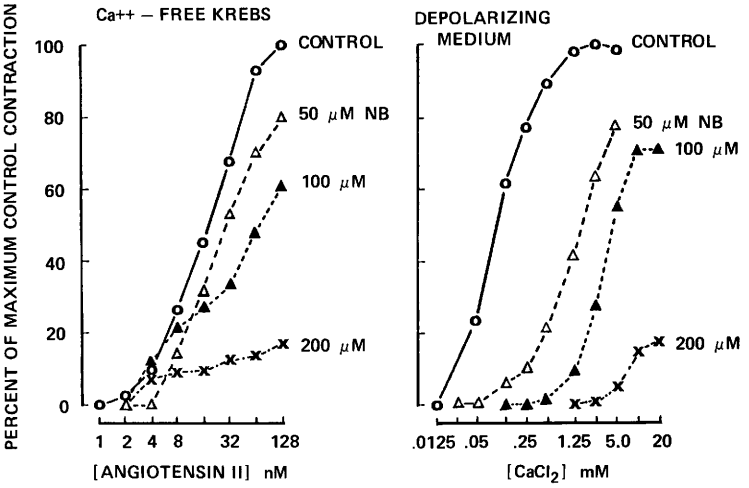

FIG. 2. Inhibition by nolinium bromide (NB) of contractions of rabbit aortic strips induced by angiotensin II (ANG II) in 0 m*M* Ca^{2+} and by $CaCl_2$ in depolarizing (40 m*M* KCl) medium. Strips were suspended in calcium-free Krebs–Ringer solution with 4.69 m*M* KCl (ANG II curves, $N = 2$–6) or 40 m*M* KCl ($CaCl_2$ curves, $N = 4$) at 37°C under 2 g tension. Contractions in the absence or presence of NB were induced by cumulatively increasing concentrations of ANG II or $CaCl_2$. Maximum control contractions were 0.5–1.3 g for ANG II and 2.6–4.2 g for $CaCl_2$.

induced contractions made use of both extracellular Ca^{2+} and a loosely bound Ca^{2+} pool in this tissue. Our finding that NB inhibited ANG II-induced contractions equally under normal and calcium-free conditions suggests that the compound is not interfering with calcium movements through plasma membrane channels.

The conclusion that calcium movements are not directly involved in NB's inhibitory

TABLE III. RECOVERY OF RABBIT AORTIC STRIP CONTRACTILE RESPONSE TO VARIOUS AGONISTS AFTER EXPOSURE TO NOLINIUM BROMIDE

Agonist	Maximum nolinium bromide concn (μ*M*)	Number of tissues	Percentage recovery of maximum contractile response (mean ± SE)
Angiotensin II in 2.5 m*M* Ca^{2+}	100	3	88.3 ± 1.8
	200[a]	3	81.3 ± 3.0
Angiotensin II in Ca^{2+}-free buffer	50	5	99.3 ± 7.1
	100	2	82.4 ± 2.2
	200	2	46.4 ± 21.4
$CaCl_2$ in 40 m*M* KCl	50	2	112 ± 1.0
	100	2	98.5 ± 3.3
	200	2	91.6 ± 0.8
Norepinephrine	200[a]	3	82.4 ± 3.2

Note. Nolinium bromide (NB) was washed from tissues by several changes of the bath medium until a stable baseline of relaxation was obtained. Contractile responses to cumulatively increasing concentrations of the indicated agonist were obtained. Maximum contractile tension was then expressed as a percentage of the same tissue's tension before exposure to NB. Time-related changes in a concurrently run control strip not exposed to NB were used to correct treated strip recovery data (see Table I).

[a] These strips had previously been exposed to 50 and 100 μ*M* NB.

action is supported by the norepinephrine results. Ackerly *et al.* (8) observed that norepinephrine-induced contractions of rabbit aortic strips were largely unaffected by verapamil, SKF-525A, or use of calcium-free buffer. Since NB does inhibit norepinephrine-induced contractions, NB is not acting like a calcium-channel blocker of the verapamil or SKF-525A type. It was further found that both the initial rate of contraction (phasic component) and the maximum contractile force (tonic component) of norepinephrine-induced contractions were equally inhibited by NB. The former depends on intracellular calcium supplies and the latter on extracellular calcium (9–12). Therefore, NB's effect is apparently not directed specifically at either of these pools of calcium.

In using a "calcium-free" buffer in the absence of a chelating agent such as EGTA, it is recognized that a low level of calcium remains available to the tissue from the external medium. Measurement of this residual Ca^{2+} with a calcium-selective electrode indicates that the concentration is less than 10^{-5} *M*. Could NB be interfering with a role of this residual Ca^{2+}? This is unlikely, since such an action would be expected to show up as differential inhibition of the tonic and phasic components of norepinephrine-induced contractions. Furthermore, NB inhibits with similar potency contractions induced by much higher (millimolar) concentrations of $CaCl_2$ which should overwhelm any inhibition of the effect of residual micromolar Ca^{2+}. It is also noted that the use of calcium-free buffer clearly shifted the nature of the ANG II-induced contractions in that maximum tension was reduced over 50% (Table I). Furthermore, time-related loss of responsiveness to ANG II, not seen in normal medium, was observed in calcium-free buffer. Nevertheless, NB inhibited such ANG II-induced contractions with equal potency as compared to normal calcium conditions. If NB were affecting a loosely bound Ca^{2+} pool, one might expect that NB's effect would change as the contribution of this pool were changed. However, NB's interference with the action of residual Ca^{2+} in "calcium-free buffer" cannot be entirely excluded.

These results suggest, but do not prove, that NB is not acting as a calcium channel blocking agent and does not interfere with internal Ca^{2+} stores directly. The salient feature, in both gastrointestinal and vascular smooth muscle, of NB's inhibition of contraction is lack of specificity for the agonist being used to induce the contraction. Some common feature of their stimulation of the muscle cell must be interfered with by NB. If NB were interacting with a membrane component, it's nonselective interference with an octapeptide, a cation, and a catecholamine in vascular smooth muscle, and with acetylcholine and histamine in gastrointestinal smooth muscle (2), could be accounted for. The surface membrane site of action would also be consistent with the polar nature of NB, which makes significant intracellular accumulation unlikely. The reversibility of NB's inhibition may also reflect a surface site of action. It would be of interest to study directly the membrane interaction of NB by use of membrane-specific fluorescent probes.

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