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THE IRREVERSIBLE INHIBITORY EFFECTS OF DIBENAMINE ON THE NORADRENALINE-, K- AND Ba-INDUCED CONTRACTIONS OF RAT'S ISOLATED VAS DEFERENS, PARTICULARLY IN RELATION TO Ca*

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Indexing Words
rat vas deferens; noradrenaline; K; Ba; irreversible inhibition by dibenamine; α-blockade; Ca mobilization; concentration-action curve; spare receptor

Kouhei OGINO. *The Irreversible Inhibitory Effects of Dibenamine on the Noradrenaline-, K- and Ba-Induced Contractions of Rat's Isolated Vas Deferens, Particularly in Relation to Ca.* Kobe J. Med. Sci. 22, 139-151, September 1976——Actions of dibenamine (DB) on the adrenergic receptor site and the non-receptor sites were investigated in relation to Ca in the isolated rat vas deferens by an observation of its effect on the contractile responses to noradrenaline (NA), potassium (K) and barium (Ba). In this preparation, contractile responses to NA, K and Ba are all initiated by the release and influx of Ca, although a direct stimulation to the contractile elements of muscle is partially concerned with the Ba-induced contraction. Storage sites of Ca in the cell membrane are distinguished into three divisions, the first, the second and the third, which contains loosely-, less loosely- and tightly-bound Ca, respectively. K releases Ca from the first, NA releases Ca from the second and Ba releases Ca from all of the three divisions to elicit contraction. DB inhibited irreversibly the contraction by NA in high preference to that by K or Ba. Against DB-induced irreversible inhibition, the NA-contration was selectively protected by a high concentration of NA or tolazoline, whereas the K- and Ba-contractions were selectively protected by a high concentration of Ca. Contractile response to Ba initiated at 3 min after the removal of Ca from bath media was irreversibly potentiated by a low concentration but irreversibly reduced by a high concentration of DB. In addition, contractile response to K in the same condition was irreversibly reduced by DB. Contractile response to exogenous Ca on assistance with K in Ca-free media was also reduced irreversibly by DB. On the other hand, the residual contraction by Ba in Ca-free media, which is due to a direct stimulation to the muscle contractile element, was not modified by the treatment with DB. From these results, it is suggested that the irreversible inhibi-

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tion by DB on the contractile response to NA may be due to the blockade of α-adrenergic receptor, whereas the irreversible inhibition by DB on the contractile responses to K and Ba may be attributable to the interference with the release and influx of Ca, although the release of Ca induced by Ba may be irreversibly accelerated by a low concentration of DB, and that the influence of DB on the muscle contractile elements may be ruled out. Discussion was made about the irreversible effect of DB on the NA-, K- and Ba-contrations analysed by the concentration-action curve.

INTRODUCTION

It is well known that β-halogenoethylamines exhibit an irreversible blocking effect on the adrenergic α-receptor. Further it has been considered that these agents also inhibit irreversibly the serotonergic,[3, 6, 17, 14] histaminergic,[3, 6, 7, 14] and cholinergic,[3, 6, 7, 14, 22] receptor sites, so that they have been utilized to determine the presence of "spare receptor". However, several lines of evidence[17, 18, 19] have indicated that β-halogenoethylamines affect not only the receptor site but also the mobilization of Ca. Furthermore, by the study in isolated rat vas deferens, Swamy and Triggle[21] presented a hypothetical model for the adrenergic α-receptor that was described as norepinephrine recognition site which is obligatorily linked with the Ca^{++}-site, the site concerned with the binding or mobilization of Ca, and they concluded that β-halogenoethylamines after their washout produced a long-lasting blockade of the norepinephrine-induced contraction by inhibiting the Ca^{++}-site rather than the α-receptor.

In the present study, to challenge the above view of Swamy et al.[21] by using the same preparation as employed by them but a different method of analysis from theirs, the antagonisms of dibenamine (DB), one of β-halogenoethylamines, were compared to the receptor stimulating drug, noradrenaline (NA) and to the non-receptor stimulating drugs, potassium (K) and barium (Ba), particularly in relation to Ca.

Preparatory research was also carried out on the mechanisms of contractile responses to NA, K and Ba in relation to the mobilization of Ca.

MATERIALS AND METHODS

Mature male rats, weighing approx. 180-250 g., were used. The animals were sacrificed by a blow on the neck and the vas deferens was excised. Strips of the vas deferens 3-4 cm long were fixed vertically in the 30 ml bath medium containing (g/l) NaCl 9.0, KCl 0.42, $CaCl_2$ 0.24, glucose 1.0 and $NaHCO_3$ 0.4 (pH 7.2—7.4). Isotonic contractions were recorded on a smoked paper with a magnification of approx. 10 times. The bath medium was maintained at $27.0\pm1.0°$ C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. The Ca-free bath medium was prepared in the same way except that $CaCl_2$ was omitted and osmotic adjustment was not made. Preparations were allowed to equilibrate in the bath medium for one hour before investigations were begun. Drug solutions were applied by dropping directly into the bath medium and their final concentrations are expressed in g/ml

of the salts unless otherwise stated.

If not otherwise specified, dibenamine was applied 15 min before and washed out 5 min after the application of contracting drugs.

Concentration-action curves of drugs were constructed according to the cumulative dose method.

Details of the experiments are described in the part of results.

Drugs employed were: potassium chloride (Yoneyama Chemicals), dl-noradrenaline hydrochloride (Sankyo), barium chloride (Yoneyama Chemicals), calcium chloride (Yoneyama Chemicals), dibenamine hydrochloride (Tokyo Kasei), 2-benzyl imidazoline hydrochloride (tolazoline) (Yamanouchi).

RESULTS

I. The Contractile Mechanism of Drugs.

1) Effect of exogenous Ca on the tone.

In the normal bath media or at 3 min after the removal of Ca from media, exogenous Ca (up to 11 mM) was without effect on the tone. However, in preparations in which K-induced contractions were lost in Ca-free bath media (70.0±3.7 min later), exogenous Ca (1.1 mM) initiated a sustained contraction on the assistance with K (3×10^{-3}), although only slightly without K. Contractile responses to exogenous Ca with K increased with increased concentration of Ca.

2) Effect of the removal of Ca from bath media on the contractile responses to noradrenaline, K and Ba.

In this experiments, concentrations used were 10^{-6} noradrenaline (NA), 3×10^{-3} K and 5×10^{-4} Ba as the approx. ED50.

Contractile responses to NA, K and Ba in normal bath media were all exponential in shape. At 3 min after the removal of Ca from normal bath media, contractions by NA and K were reduced to 15.6±4.2% (N=6) and 41.4±6.3% (N=12), respectively, which were followed by the further reduction to 12.5±3.8% (N=6) and 35.8±5.8% (N=12), respectively, due to the phenomenon of "fade", as compared with those obtained in normal media. On the other hand, influence of the removal of Ca on the contractile response to Ba was multiple and had an seasonal variation. In the season from April to November, removal of Ca usually potentiated the contraction by Ba to 161.2±14.8% (N=12), as compared with that in normal media, although a marked fade rarely occurred after this potentiation, resulting in the final reduction to 56.5±8.1% (N=6). And in the period from December to March, contraction by Ba was reduced to 80.4±2.6% (N=6) and followed by the final reduction to 20.6±3.9% (N=6) due to fade, as compared with that in normal media. Examples of these results are shown in Fig. 1.

In the following experiments are described the results concerned with the usual case of Ba-contraction obtained in the season from April to November.

In Ca-free media, contractile responses to NA, K and Ba gradually decreased by repeated treatment and the contraction by K disappeared 70.0±3.7 min (N=8) later, while that by NA disappeared 152.5±3.7 min (N=8) later. The contraction by Ba further decreased but 192.5±3.7 min (N=8) later it remained constant at the

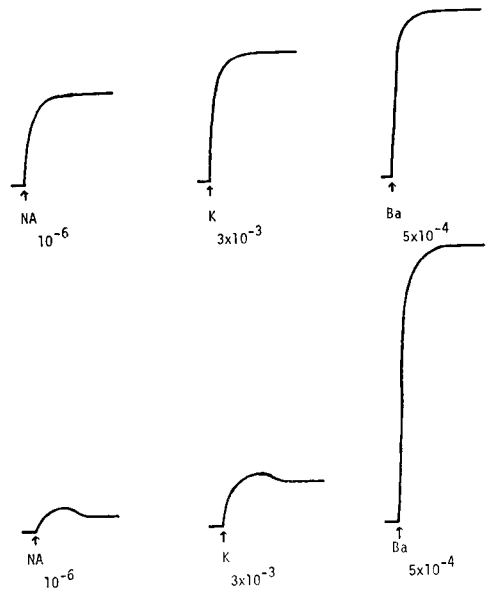

Fig. 1 Contractions by noradenaline (NA), K and Ba in normal bath media (upper) and in Ca-free bath media 3 min after removal of Ca (below).

magnitude of 18.2±3.6% (N=8), as compared with that in normal media, that is, "residual contraction by Ba" was obtained. The time required for the disappearance of K- or NA-induced contraction in Ca-free media was markedly shortened to 27.5± 3.7 min (N=8) and 87.5±3.7 min (N=8), respectively, by the prior treatment with Ba (2×10^{-3}, ED100)-induced contraction followed by washout, but not affected by the prior treatment with NA (4×10^{-6}, ED100) or K (5×10^{-3}, ED100), respectively, followed by washout.

The time required for the disappearance of drug-induced contractions mentioned here was determined by the initial loss of contractile responses, when the drugs were repeatedly applied for 5 min at 3 min and at every 20 min interval after the Ca removal from normal media. Similar procedures were used in determining the time required for occurrence of the residual contraction by Ba.

II. Effect of Dibenamine.

1) Effect of dibenamine on the tone.

A high concentration (10^{-3}) of dibenamine (DB) produced a reversible contraction in normal media. In Ca-free media, the DB (10^{-3})-induced contraction was equal in magnitude 3 min later to that in normal media, but gradually decreased and finally disappeared in parallel with the disappearance of K-induced contraction.

2) Influence on the contractile responses to NA, K and Ba.

In normal media, DB produced an irreversible inhibition on the contractile responses to NA and K from the concentrations of 10^{-8} and 3×10^{-4}, respectively,

and abolished them irreversibly in the concentrations of 2×10^{-7} and 10^{-3}, respectively. The Ba-induced contraction was irreversibly potentiated by the concentration 5×10^{-7} -5×10^{-4} but irreversibly reduced by 10^{-3} of DB. The results are summarized in Table 1.

Table 1 Influence of dibenamine (DB) on the noradrenaline (NA)-, K- and Ba-induced contractions in normal bath media.

		NA	K	Ba
Control		100 (N=6)	100 (N=6)	100 (N=6)
After treatment with DB	10^{-8}	80.1±2.4 (N=6)	···	···
	10^{-7}	3.4±0.3 (N=6)	···	···
	2×10^{-7}	0 (N=6)	···	···
	10^{-6}	···	···	132.3±3.6 (N=6)
	5×10^{-4}	···	42.6±3.1 (N=6)	109.1±1.0 (N=6)
	10^{-3}	···	0 (N=6)	40.2±1.6 (N=6)

Values compared with the contraction height (100%) by approx. ED50 of agonists are means±S.E. (%). N: number of preparations. ···: no experiment.

In Ca-free media at 3 min after the Ca removal, K-induced contraction was irreversibly reduced by the concentration 3×10^{-4}—5×10^{-4} and was irreversibly disappeared by 10^{-3} of DB, whereas the Ba-induced contraction was irreversibly potentiated by the concentration 10^{-6}—5×10^{-4} and was irreversibly depressed by 10^{-3} of DB. The results are summarized in Table 2.

Table 2 Influence of dibenamine (DB) on the K- and Ba-induced contractions in Ca-free bath media 3 min after the removal of Ca.

		K	Ba
Normal bath media		100 (N=6)	100 (N=6)
No treatment in Ca-free bath		46.8±2.4 (N=6)	160.1±3.8 (N=6)
Treatment with DB in Ca-free bath media	10^{-6}	···	194.3±4.6 (N=6)
	3×10^{-4}	24.1±1.8 (N=6)	172.0±2.4 (N=6)
	10^{-3}	0 (N=6)	55.5±2.7 (N=6)

Values compared with the contraction height (100%) by approx. ED50 of agonists in normal bath media are means±S.E. (%). N: number of preparations. ···: no experiment.

The residual contraction by Ba in Ca-free media was not modified by the treatment with DB even in the concentration of 10^{-3}.

3) *Influence on the contractile response to exogenous Ca.*

In preparations in which the K-induced contraction was lost in Ca-free media, the contraction induced by 4.4 mM (approx. ED50) of exogenous Ca on the assistance

with K (3×10^{-3}) was irreversibly reduced by the treatment with DB 10^{-3}. Example is shown in Fig. 2.

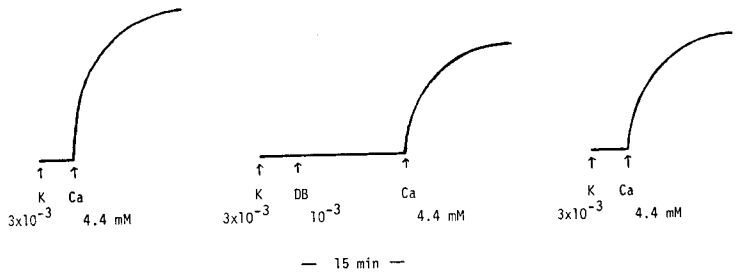

Fig. 2 Influence of dibenamine (DB) on the Ca-induced contraction in Ca-free, K-depolarizing bath media.

4) Protection experiments against the irreversible effect of dibenamine.

The irreversible inhibitory effect of DB (2×10^{-7}) on the NA-induced contraction was protected by the combined treatment with a high concentration of tolazoline (4×10^{-5}) or NA (1.5×10^{-5}) but not by the combined treatment with a high concentration of K (1.2×10^{-2}), Ba (4×10^{-3}) or exogenous Ca (11 mM). On the other hand, the irreversible inhibitory effect of DB (5×10^{-4}) on the K-contraction, the irreversible potentiating effect of DB (10^{-6}) on the Ba-contraction and the irreversible inhibitory effect of DB (10^{-3}) on the Ba-contraction were all protected by the combined treatment with exogenous Ca (8.8 mM), but not by the combined treatment with tolazoline (4×10^{-5}), K (1.2×10^{-2}), Ba (4×10^{-3}) or NA (1.5×10^{-5}). Examples of protection experiments are illustrated in Figs. 3 and 4 and the results are summarized in Table 3.

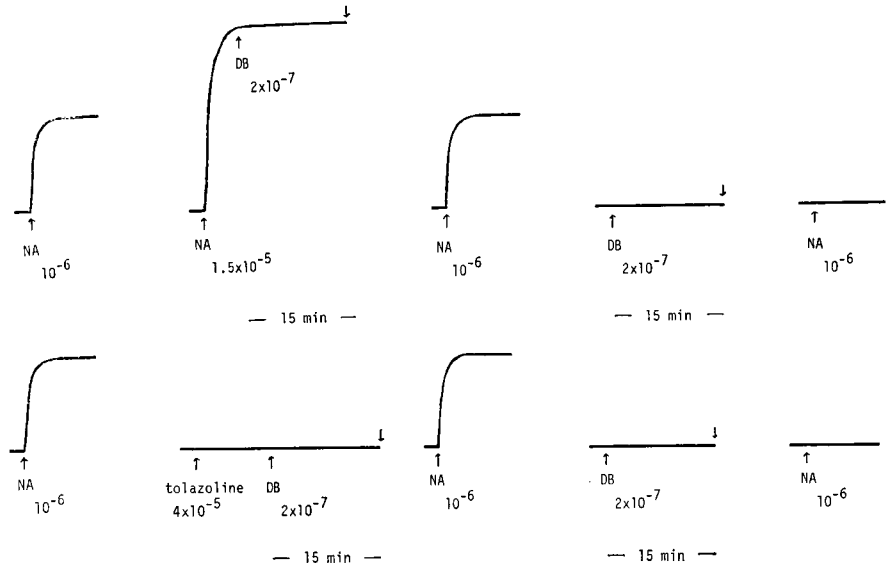

Fig. 3 Protection by noradrenaline (NA) and tolazoline against dibenamine (DB)-induced irreversible inhibition of NA-induced contraction.

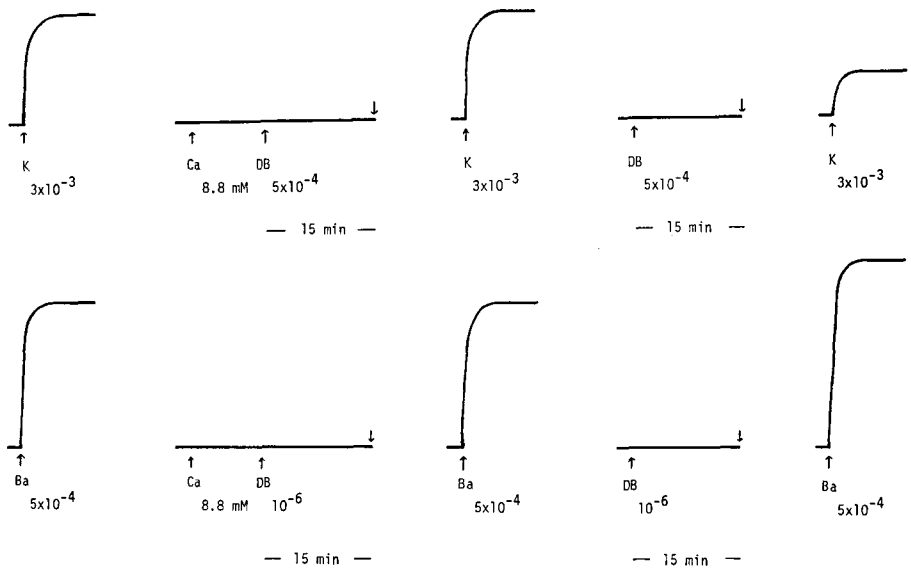

Fig. 4 Protection by exogenous Ca against dibenamine (DB)-induced irreversible inhibition of K- and Ba-induced contractions.

Table 3 Results of protection experiments by pretreatment with the drugs tested against the irreversible effect of dibenamine (DB).

Agonists / Concentration of DB used / Pretreatment with	NA	K	Ba	
	2×10^{-7}	5×10^{-4}	10^{-6}	10^{-3}
none	100 (N=6)	100 (N=6)	100 (N=6)	100 (N=6)
DB	0 (N=6)	44.6±3.8 (N=6)	130.2±5.4 (N=6)	37.5±6.3 (N=6)
Ca+DB	0 (N=6)	93.4±4.3*(N=6)	102.1±3.2*(N=6)	97.1±2.2*(N=6)
NA+DB	96.2±2.6*(N=6)	48.1±5.7 (N=6)	123.9±3.3 (N=6)	34.6±3.3 (N=6)
K+DB	0 (N=6)	46.5±2.9 (N=6)	131.1±4.6 (N=6)	36.7±2.9 (N=6)
Ba+DB	0 (N=6)	49.8±4.8 (N=6)	132.4±6.8 (N=6)	39.8±3.2 (N=6)
tolazoline+DB	95.7±3.4*(N=6)	41.6±3.7 (N=6)	122.1±2.9 (N=6)	35.5±4.1 (N=6)

Magnitudes of noradrenaline-, K- and Ba-induced contractions without pretreatment are taken as 100%. N : number of preparations. *: Protective effects are statistically significant ($p<0.05$).

5) *Analysis of the irreversible effect of dibenamine by means of the concentration-action (C—A) curve.*

DB was applied 15 min before and washed out 20 min after the application of contracting drugs. Incubation with DB produced a shift of the log C-A curves for the contracting drugs tested with a slight recovery observable with the lapse of time, but this recovery was lost 90 min later and then remained in the "final state". This final state is hereafter referred to as the irreversible effect of DB.

The concentration of DB required to irreversibly move the log C—A curve of NA to the most marked parallel shift to the right ($d=0.43\pm0.01$ log unit, $N=5$) was 3×10^{-8}, since the downward shift was observed in addition with a higher concentration (Fig. 5). The log C—A curve of K was shifted downwards irreversibly by

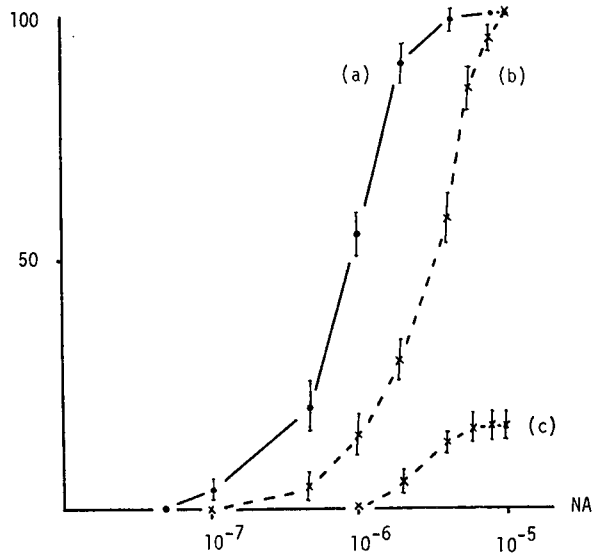

Fig. 5 Effect of dibenamine (DB) on the concentration-action curve of noradrenaline (NA).
(a): control (N=5). (b) and (c): after washout of DB 3×10^{-8} (N=5) and 2×10^{-7} (N=5), respectively.
Abscissa; concentration of NA in log scale.
Ordinate; percentage of maximum contraction.

$3\times10^{-4}-5\times10^{-4}$ and still more by 10^{-3} of DB. On the other hand, the log C—A curve of Ba was irreversibly shifted to the left in parallel by a low concentration (10^{-6}) and was irreversibly shifted to the left and downwards by intermediate concentrations ($3\times10^{-6}-10^{-4}$) of DB, whereas it was irreversibly and strongly shifted downwards by a high concentration (10^{-3}) of DB (Fig. 6). The log C—A curve of exogenous Ca in Ca-free, high K-depolarizing media was irreversibly shifted downwards by 10^{-3} of DB.

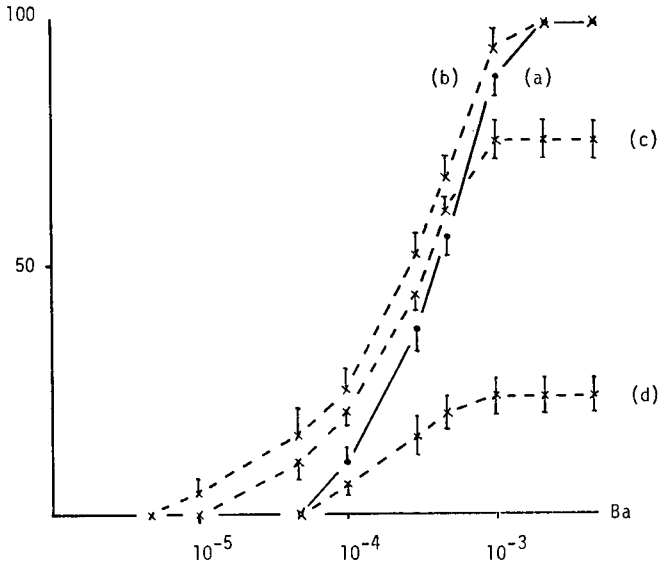

Fig. 6 Effect of dibenamine (DB) on the concentration-action curve of Ba.
(a) : control (N=6), (b), (c) and (d) ; after washout of DB 10^{-3} (N=6), 5×10^{-5} (N=6) and 10^{-3} (N=6), respectively.
Abscissa; concentration of Ba in log scale.
Ordinate; percentage of maximum contraction.

DISCUSSION

I. The Contractile Mechanism of Drugs.

The present study demonstrated that at 3 min after the removal of Ca from normal bath media NA- and K-induced contractions were significantly reduced as compared with those in normal media. Thus, it is suggested that contractile responses to NA and K are initiated not only by the release of Ca but also the influx of Ca. On the other hand, the Ba-induced contraction was either potentiated or reduced by the 3 min removal of Ca, depending largely on the seasonal variation. This implies that the contractile response to Ba is also attributable to release and influx of Ca and the potentiation of Ba-contraction is due to the acceleration of Ca release.

The results obtained here concerning the NA- and K-induced contractions are consistent with the reports by Chang et al.[5] and Jains et al.[9] in rat vas deferens, and the potentiation of Ba-induced contraction is in agreement with the results of Araki et al.[2] in rabbit's ileum.

In preparations which were incubated in Ca-free bath media, contractile responses to drugs gradually decreased by repeated treatments, and the K- and NA-induced contractions disappeared about 70 min and about 150 min later, respectively, whereas the residual contraction by Ba was obtained about 190 min later. In addition, the

time required for disappearance of the K- or NA-induced contraction in Ca-free media was markedly shortened by prior treatment with Ba (2×10^{-3}, ED100)-induced contraction followed by washout, although it was not affected by the prior treatment with NA (4×10^{-6}, ED100)-induced or K (5×10^{-3}, ED100)-induced contraction, respectively, followed by washout. From these findings, the following is suggested; (1) Storage sites of Ca in the cell membrane of this preparation are distinguished into three divisions, the first, the second and the third, which contains loosely, less loosely and tightly-bound Ca, respectively; K releases Ca from the first, NA releases Ca from the second and Ba releases Ca from all of the three divisions for producing contraction. (2) The direct stimulation to muscle contractile elements is also partially concerned with the contractile mechanism of Ba.

The above presumption of the division in Ca stores is consistent with the similar view of Hudgins and Weiss[8] in rabbit's aorta strip and of Jurkiewiczs et al.[10] in rat vas deferens. There are discrepancies between the reports concerning the contractile mechanism of Ba on the smooth muscle, such as, (1) the indirect action mediated by Ca release[11, 12, 15, 20] (2) the direct stimulation to the contractile elements[16] and (3) the combined action of (1) and (2).[1] The conclusion obtained in this study coincides with the view of (3).

II. Effect of Dibenamine.

1) Effect of dibenamine on the tone.

DB 10^{-3} produced a reversible contraction in normal media, whereas in Ca-free media, the DB (10^{-3})-induced contraction was similar in magnitude to that in normal media 3 min later but gradually decreased and finally disappeared in parallel with the loss of K-contraction. From this, it is suggested that DB releases Ca from the K-sensitive Ca store for producing contraction.

2) Influence of dibenamine on the contractile responses to drugs.

In normal media, DB produced an irreversible inhibitory effect on the NA-induced contraction in high preference to the K- and Ba-induced contractions. On the other hand, DB inhibited the K-contraction irreversibly, whereas the Ba-contraction was irreversibly potentiated by a low concentration but inhibited irreversibly by a high concentration of DB. In addition, in Ca-free media at 3 min after Ca removal, the irreversible action of DB on the K- and Ba-contractions was similar to that in normal media. From these results, it can be considered that the NA-contraction is irreversibly inhibited by the blockade of adrenergic α-receptor, one of the well known action of DB, whereas the release of Ca by K is irreversibly reduced by DB, and the release of Ca by Ba is accelerated by a low concentration but reduced by a high concentration of DB.

Inhibitory effect of DB on the K-induced contraction is similar to the suggestion of Shibata et al. in rabbit's aorta[17] and guinea pig *taenia coli*[18] and the report of Araki et al.[2] in rabbit's ileum. In addition, the inhibitory effect of DB in a high concentration on the Ba-indnced contraction is similar to the report of Araki et al.[2] in rabbit's ileum. However, the accelerating action of DB in a low concentration on the Ba-induced Ca release has not been reported to date.

Furthermore, the residual contraction by Ba in Ca-free media was not modified by a high concentration (10^{-3}) of DB. Thus, the direct effect of DB on the contractile

element of muscle is ruled out.

3) Influence of dibenamine on the contractile response to exogenous Ca.

The contractile response to exogenous Ca in a Ca-free, high K-depolarizing medium was irreversibly reduced by a high concentration (10^{-3}) of DB. From this, it is presumed that the irreversible inhibitory action of DB in a high concentration on the K- and Ba-contractions in normal media is partially due to irreversible inhibition of the Ca influx caused by K and Ba.

4) Protection experiments against the irreversible effect of dibenamine.

Protective effect against the irreversible inhibitory effect of DB on the NA-induced contraction was selectively observed by the combined treatment with NA or tolazoline (Table 3). This finding further supports the suggestion as stated above that the irreversible inhibitory effect of DB on the NA-contraction is mediated through the blockade of adrenergic α-receptor, and does not coincide with the view of Swamy and Triggle suggested in rabbit's aorta[9] and in rat vas deferens[13, 21] that this effect is due to inhibition of the Ca^{++}-site rather than the α-receptor.

On the other hand, protective effect against the irreversible action of DB on the K- and Ba-induced contractions was selectively obtained by the combined treatment with Ca (Table 3). This result suggests that the irreversible influence of DB on the K- and Ba-induced contractions is a result of its effect on Ca^{++}-site in agreement with the suggestions by Shibata et al.[17, 18] and by Araki et al.[2]

5) Analysis of the irreversible effect of dibenamine by means of the concentration-action (C—A) curve.

The irreversible effect of DB on the log C—A curve of NA occurred in high preference to that of K or Ba. The concentration of DB required to move the log C—A curve of NA to the maximum parallel shift to the right was 3×10^{-8}, since the downward shift was observed in addition with a higher concentration of DB. In contrast to this, the log C—A curve of K was irreversibly shifted downwards by DB in conecntrations of $3\times10^{-4}-10^{-3}$, while the log C—A curve of Ba was irreversibly shifted to the left in parallel by a low concentration (10^{-6}), to the left and downards by intermediate concentrations ($3\times10^{-6}-10^{-4}$) and downwards by a high concentration (10^{-3}) of DB. These findings indicate that the irreversible parallel shift of the log C—A curve of NA to the right by DB may be due to the presence of spare receptors but not through the action on Ca^{++}-site as proposed by Swamy et al.[21], whereas the irreversible action of DB on the log C—A curves of K and Ba may be due to the action on Ca^{++}-site. It is also suggested that the downward shift of log C—A curves of K and Ba by DB is mainly due to the inhibition of Ca release and in addition in the case of the concentration 10^{-3} of DB partially due to the inhibition of Ca influx, whereas the shift of the log C—A curve of Ba by DB to the left is due to accelerated release of Ca.

Ariëns[4] reported the absence of spare receptors in rat vas deferens from the observation that the log C—A curve of NA was shifted only downwards by DB. The result obtained in the present study does not coincide with this report.

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