Mechanism of Tachyphylaxis on the Inhibitory Effect of Manganese on Agonist-Induced Contractions in the Isolated Vas Deferens of the Guinea Pig

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In the isolated vas deferens of the guinea pig, the inhibitory effect of manganese (Mn^{2+}) on contractions induced by norepinephrine decreased when the contractions were induced repeatedly in the presence of Mn^{2+} . This phenomenon, "tachyphylaxis" to the inhibitory effect of Mn^{2+} , was minimally observed when contractions were induced by acetylcholine. Contractions induced by acetylcholine as well as by norepinephrine in a Mn^{2+} -free medium were augmented in preparations in which manganese had accumulated intracellularly by repetitive applications of high potassium in the presence of Mn^{2+} . The magnitudes of their augmentation were dependent on the manganese contents of the preparations. The augmented contractions were remarkably resistant to the inhibitory effect of Mn^{2+} applied to the medium. These results suggest that intracellular manganese augments the contractions and this augmentation results in an apparent decrease in the inhibitory effect of Mn^{2+} in the medium.

Key words manganese; norepinephrine; acetylcholine; tachyphylaxis; guinea pig vas deferens

Manganese (Mn^{2+}) has been known to inhibit Ca influx through the membrane and hence to inhibit the contractions of smooth muscles.¹⁾ However, under some conditions, Mn^{2+} initiates or accelerates mechanical responses in various smooth muscles and the myocardium.²⁾ These effects suggested that Mn^{2+} was a partial agonist mimicking calcium or that Mn^{2+} acted differently at different sites, resulting in increasing intracellular calcium ion concentration.²⁾ Recently, we found that the inhibitory effect of Mn^{2+} on contractions induced by potassium (K^+) decreased when the contractions were repeatedly evoked in the presence of Mn^{2+} . We designated this as "tachyphylaxis" to the inhibitory effect of Mn^{2+} .³⁾ It was also shown that Mn^{2+} entered into smooth muscle cells through diltiazem-sensitive calcium-channels activated by K^+ , norepinephrine (NE) and acetylcholine (ACh), and accumulated in the cells.⁴⁾ The most potent agonist stimulating manganese accumulation was K^+ . ACh was least effective among the three agonists. Since the development of "tachyphylaxis" was correlated with increases in the manganese content of the preparations, it was assumed that the augmenting effect of the intracellularly accumulated Mn^{2+} counteracted the inhibitory effect of extracellular Mn^{2+} and this resulted in the "tachyphylaxis."³⁾

In this study, the appearance of "tachyphylaxis" has been examined using NE and ACh, which cause contractions through a different mechanism from that of K^+ . Further, to confirm the above hypothesis about the mechanism of "tachyphylaxis," the augmenting effect of intracellular Mn^{2+} has also been examined.

MATERIALS AND METHODS

Male guinea pigs of the Hartley strain weighing 350 to 550 g were sacrificed by a blow to the neck. Vasa deferentia were isolated and connective tissues were removed. A strip of 8 to 10 mm in length was prepared from the epididymal portion of a vas deferens and was mounted under a 0.3 g

load in an organ bath containing aerated HEPES–Locke–Ringer solution (normal medium) of the following composition (mm): NaCl, 154; KCl, 5.6; $MgCl_2$, 2.1; $CaCl_2$, 2.2; glucose, 2.8; HEPES (*N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid), 4.4, adjusted to pH 7.4 at 30 °C with 1 N NaOH. Isotonic high- K^+ medium (100 mm K^+) was prepared by replacing 94.4 mm of the NaCl in the normal medium with an equimolar concentration of KCl.

After equilibration in the normal medium for 60 min, isotonic triphasic contractions in response to 10 μ M NE and 10 μ M ACh were recorded for 5 min with 12.5 times magnification with an isotonic transducer (Nihon Kohden, Model TD-112S). Magnitudes of the phasic and tonic components of these contractions were, respectively, 100% and about 90% of the corresponding components of the maximal contractions induced by 100 μ M of each agonist. Only those preparations which exhibited reproducible contractions to the three successive applications of either NE or ACh were used. The third contraction to each agonist was taken as "the control contraction." The magnitude of each component of the contraction was measured from the resting level just before each application of an agonist. Unless otherwise stated, each component of "the control contraction" was normalized as 100%.

Mn^{2+} (0.21–2.1 mm) was applied 10 min prior to an application of the agonists. In some experiments, 100 mm K^+ was applied for 5 min by substitution of the bathing medium with a high- K^+ medium containing Mn^{2+} . The application of K^+ in the presence of Mn^{2+} was repeated 9 times once every 20 min in each preparation. This procedure, which was performed in order to accumulate manganese in the smooth muscle cells, was designated as the "Mn-loading procedure." As has been shown, the manganese content of the Mn-loaded preparations did not decrease significantly throughout the present experiments.⁴⁾ Each agonist was applied every 20 min, except for the first application of NE and ACh after the "Mn-loading procedure," which was done 30 min after

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the completion of the procedure.

Results were expressed as mean values $\pm$ S.E.M. Statistical analysis was made using the paired *t*-test. A *p* value of less than 5% was considered to be significant.

The drugs used were: *L*-NE (Nacalai Tesque), ACh chloride (Daiichi-seiyaku), manganese chloride (Kanto Kagaku). NE was dissolved in 0.1 N HCl and diluted with 0.1% NaHSO₃ solution. ACh and MnCl₂ were dissolved in de-ionized and distilled water.

RESULTS

Effects of Manganese on the Contractions Induced by NE or ACh

NE or ACh caused a contraction composed

of three components, namely the first small transient phase (prephasic component), the second large rapid phase (phasic component), and the third sustained phase (tonic component). Magnitudes of the prephasic components induced by both agonists were less than 20% of the respective phasic components. Ratios of tonic to phasic components of NE- and ACh-induced contractions were 0.55 ± 0.03 ($n=26$) and 0.46 ± 0.03 ($n=32$), respectively. When NE was applied repeatedly every 20 min, the phasic and tonic components of the contractions were reproducible (Fig. 1a). However, both the phasic and tonic components of the ACh-induced contractions decreased linearly, and the magnitudes of these components of the 15th contraction were $92.4 \pm 2.4\%$ and $74.5 \pm 3.0\%$ of

Fig. 1. Effects of Manganese on the Contractions Repeatedly Induced by 10 μ M NE

The concentrations of Mn²⁺ used in the treatment were 0.21 mM (a) and 0.37 mM (b). Both the phasic (○) and tonic (●) components were shown as a percentage of each component of "the control contraction" (C). The prephasic component (△) was shown as a percentage of the phasic component of "the control contraction". In (a), the reproducibility of norepinephrine-induced contractions was shown (solid line: phasic component, dotted line: tonic component, dashed line: prephasic component). Results represent the mean $\pm$ S.E.M. (a: $n=6$, b: $n=7$).

Fig. 2. Effects of Manganese on the Contractions Repeatedly Induced by 10 μ M ACh

The concentrations of Mn²⁺ used in the treatment were 0.21 mM (a) and 0.37 mM (b). Both the phasic (○) and tonic (●) components were shown as a percentage of each component of "the control contraction" (C). The prephasic component (△) was shown as a percentage of the phasic component of "the control contraction". In (a), the reproducibility of acetylcholine-induced contractions was shown (solid line: phasic component, dotted line: tonic component, dashed line: prephasic component). Results represent the mean $\pm$ S.E.M. ($n=8$).

each component of "the control contraction," respectively ($n=6$, Fig. 2a). Magnitudes of the prephasic component of NE- and ACh-induced contractions were varied and did not show any time-dependency. Resting levels hardly changed during repetitive applications of the agonists.

Mn^{2+} (0.21, 0.37 mM) inhibited NE-induced contractions in a dose-dependent manner. The prephasic component was inhibited not by the first application but by subsequent applications of Mn^{2+} . Mn^{2+} inhibited the tonic components more than the phasic components (Fig. 1). At the first application of 0.21 mM Mn^{2+} , the phasic and tonic components of NE-induced contractions were inhibited by $7.3 \pm 1.5\%$ and $52.2 \pm 5.5\%$, respectively (Fig. 1a, $n=6$). A higher concentration of Mn^{2+} (0.37 mM) inhibited these components by $76.8 \pm 7.0\%$ and $79.6 \pm 2.7\%$, respectively (Fig. 1b, $n=7$). When NE-induced contractions were repeatedly evoked in the presence of Mn^{2+} , the magnitude of the inhibitory effects of Mn^{2+} gradually decreased. At the 9th contraction, the inhibitory effect of Mn^{2+} fell to $16.4 \pm 4.9\%$ (on the tonic component, with 0.21 mM Mn^{2+}), $17.7 \pm 2.6\%$ and $30.3 \pm 2.1\%$ (on the phasic and tonic component, with 0.37 mM Mn^{2+} respectively). Thus, it was shown that the "tachyphylaxis" appeared in NE-induced contractions as well as in K^+ -induced ones.³⁾ Resting levels were minimally affected by repetitive applications of either concentration of Mn^{2+} .

ACh-induced contractions were also inhibited by Mn^{2+} in a manner similar to the NE-induced contractions (Fig. 2). However, the appearance of "tachyphylaxis" was minimal even if ACh-induced contractions in the presence of Mn^{2+} were repeated up to 15 times. Even at a concentration of Mn^{2+} as high as 0.37 mM, the decrease in the inhibition by Mn^{2+} on the tonic component was slight (from $87.9 \pm 3.0\%$ at 2nd application to $77.1 \pm 4.8\%$ at 15th). Thus, the degree of "tachyphylaxis" was markedly less than that in the NE-induced contractions.

Effects of Intracellularly Accumulated Manganese on the Contractions Induced by NE and ACh To examine whether intracellularly accumulated manganese augmented the contractions induced by the agonists, NE- and ACh-induced contractions were elicited in Mn^{2+} -loaded

preparations. As previously reported, using the "Mn-loading procedures" with 0.21 or 2.1 mM Mn^{2+} , we can prepare two kinds of Mn^{2+} -loaded preparations in which either a low ($0.69 \pm 0.03 \mu\text{mol/g}$ wet weight) or high ($3.95 \pm 0.25 \mu\text{mol/g}$ wet weight) amount of manganese is accumulated, respectively.⁴⁾

Both NE- and ACh-induced contractions were attenuated by 9 applications of K^+ without Mn^{2+} (control for "Mn-loading procedure," Fig. 3). Each component of both contractions decreased to 60 to 80% of the corresponding component of "the control contractions."

In the Mn^{2+} -loaded preparations that accumulated a low amount of manganese, contractions induced by the agonists in the Mn^{2+} -free medium were composed of three components, similarly to "the control contractions." The prephasic components of both contractions and the phasic components of the ACh-induced ones were not augmented. The phasic components of NE-induced contractions and the tonic components of both contractions were significantly augmented (Fig. 3). When the contractions were induced by the agonists in the Mn^{2+} -free medium after the Mn-loading procedure with a higher concentration of Mn^{2+} , the phasic components disappeared and the contractions were composed of prephasic and tonic components. These components of both contractions were remarkably augmented (Fig. 3). No qualitative difference in the effects of intracellularly accumulated manganese was shown between the NE- and ACh-induced contractions.

In the Mn^{2+} -loaded preparations in which high amounts of manganese were accumulated, the augmented tonic components of NE- and ACh-induced contractions were inhibited by Mn^{2+} applied to the medium in a dose-dependent manner. However, the inhibitory effect of extracellular Mn^{2+} on the augmented contractions was less than on the contractions in the Mn-unloaded preparations (Fig. 4). Both NE- and ACh-induced contractions in the Mn-unloaded preparations were eliminated by extracellularly applied 2.1 mM Mn^{2+} (Fig. 4). In contrast, magnitudes of the augmented prephasic and tonic components of NE-induced contractions that remained in the presence of extracellular Mn^{2+} (2.1 mM)

Fig. 3. Effects of Intracellular Manganese on the Contractions Induced by $10 \mu\text{M}$ NE (a) and $10 \mu\text{M}$ ACh (b)

The magnitudes of the components are shown as a percentage of their respective components of "the control contractions". A: after 9 applications of $100 \text{ mM } K^+$ without Mn^{2+} , B: after the Mn-loading procedure with $0.21 \text{ mM } Mn^{2+}$, C: after the Mn-loading procedure with $2.1 \text{ mM } Mn^{2+}$. Vertical bars indicated S.E.M. ($n=6-8$).

Fig. 4. Effects of Extracellular Manganese on the Augmented Tonic Components of the Contractions Induced by $10\ \mu\text{M}$ NE (a) and $10\ \mu\text{M}$ ACh (b)

Each tonic component was shown as a percentage of the tonic component of the contraction induced in Mn-free medium. ○, after 9 applications of $100\ \text{mM}$ K^+ without Mn^{2+} (Mn-unloaded preparation); ●, after the Mn-loading procedure with $2.1\ \text{mM}$ Mn^{2+} (Mn-loaded preparation). Results represent the mean $\pm$ S.E.M. ($n=6-8$).

were $146.9 \pm 43.6\%$ and $111.7 \pm 18.3\%$ of the corresponding component of each of "the control contractions," respectively. Similarly, the remaining prephasic and tonic components of the augmented ACh-induced contractions were $108.8 \pm 8.1\%$ and $156.4 \pm 11.5\%$ of the corresponding component of each of "the control contractions," respectively.

DISCUSSION

Mn^{2+} inhibited both NE- and ACh-induced contractions of guinea pig vas deferens in a dose-dependent manner. The inhibitory effect on NE-induced contractions was decreased according to repetition of the NE application in the presence of Mn^{2+} . "Tachyphylaxis" appeared in both the phasic and tonic components of NE-induced contractions. In contrast to the NE-induced contractions, "tachyphylaxis" minimally appeared in the ACh-induced contractions evoked repeatedly in the presence of the lower concentration of Mn^{2+} . In the presence of the higher concentration of Mn^{2+} , only the tonic component was slightly restored with repetition of the application of ACh. However, the degree of "tachyphylaxis" was clearly smaller than that in the NE-induced contractions. As previously reported, NE stimulated intracellular manganese accumulation more strongly than ACh. These results are consistent with the hypothesis about the appearance of "tachyphylaxis," namely, that intracellularly accumulated manganese augmented contractions and resulted in a counteraction to the inhibitory effect of extracellular Mn^{2+} .³⁾ If this hypothesis is correct, NE- and ACh-induced contractions would be augmented by the intracellular accumulation of manganese. In fact, as shown in the present study, the prephasic and tonic components of both NE- and ACh-induced contractions induced in a Mn^{2+} -free medium were potentiated in the Mn-loaded preparations,

and the magnitudes of potentiation appeared to be dependent on the amount of manganese in the preparations.

The degrees of "tachyphylaxis" in NE-induced contractions and the potentiation of the NE- and ACh-induced contractions in the Mn-loaded preparations were larger than that of the K^+ -induced contractions which were shown previously,³⁾ even though a similar amount of manganese was accumulated in each preparation. In contrast to the previous study, in which K^+ was used as stimuli,³⁾ resting levels changed minimally during and after the repetitions of NE- and ACh-induced contractions in the presence of Mn^{2+} . These differences might result from the difference in calcium sources used. Both NE- and ACh-induced contractions depend on both Ca^{2+} -release from intracellular calcium stores and Ca^{2+} -influx,⁵⁾ whereas K^+ -induced contractions mainly depend on Ca^{2+} -influx through voltage-dependent calcium-channels (VDCs). If intracellularly accumulated Mn^{2+} inhibits intracellular Ca^{2+} movement, or Ca^{2+} sequestration to sarcoplasmic reticulum and Ca^{2+} extrusion, it might lead to a stronger augmentation of the NE- and ACh-induced contractions depending on Ca^{2+} release. Recently, it has been suggested that the Ca^{2+} -sensitivity of contractile elements may be increased by certain agonists, including NE and ACh.⁶⁾ Thus, a small increase in $[\text{Ca}^{2+}]_i$ by intracellular Mn^{2+} would cause a stronger augmentation of NE- and ACh-induced contractions than of a K^+ -induced contraction. It is also possible that intracellular Mn^{2+} interacts with a certain step along the intracellular signal transduction pathways activated by NE and ACh, and then augments the contractions, not *via* the increase in $[\text{Ca}^{2+}]_i$. Filippini *et al.* suggested that Mn^{2+} has some sort of amplifying effect on the EGTA-resistant ACh-contraction in canine fundus smooth muscle.⁷⁾

Phasic components of both contractions disappeared in the preparations loaded with higher concentrations of Mn^{2+} . They might be masked by the tonic components that were remarkably augmented. However, the disappearance might also be due to the difference in Ca^{2+} sources on which each component depended. The phasic components, but not the prephasic or tonic components, of both contractions are mainly dependent on Ca^{2+} influx through VDCs.⁵⁾ It has been suggested that Mn^{2+} inhibits VDCs at a concentration of more than $1\ \text{mM}$.³⁾ When $2.1\ \text{mM}$ Mn^{2+} was applied to the medium during the "Mn-loading procedure," it is likely that superficially bound manganese remained even after exchanging the bathing medium to a Mn^{2+} -free medium, and that the superficial manganese suppressed VDCs.

Mn^{2+} applied to the normal medium inhibited the augmented tonic components of both contractions in a concentration-dependent manner. However, the magnitudes of these inhibitions were remarkably less than those in the corresponding contractions of preparations without Mn-loading procedures. This result is also consistent with the above hypothesis about the appearance of "tachyphylaxis."

In conclusion, "tachyphylaxis" to the inhibitory effect of Mn^{2+} also appears in NE- and ACh-induced contractions in a way similar to K^+ -induced contractions. Intracellu-

larly accumulated manganese augments contractions induced by receptor-mediated agonists, dependently on the manganese content in preparations. The counteraction of the augmenting effect of intracellular manganese against the inhibitory effect of extracellular Mn^{2+} may result in the appearance of "tachyphylaxis."

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