The Uptake of Manganese Induced by Agonists in the Isolated Vas Deferens of the Guinea Pig

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(Received October 24, 1989)

The uptake of manganese (Mn) induced by 100 mm potassium (K), 10 μM norepinephrine (NE) or 10 μM acetylcholine (ACh) was measured by atomic absorption spectroscopy in isolated vas deferens of the guinea pig. The agonists at these concentrations caused the maximal contraction of the vas deferens. The contents of Mn were increased with the repetitive treatments of Mn with K and were not significantly decreased after 1 h washing. The uptake of Mn was also stimulated by NE and ACh. The stimulation of uptake of Mn by K was the most potent while that by ACh was the smallest. The uptake of Mn was enhanced by elimination of Ca from the medium, while inhibited by the higher concentration of external Ca and by 2.1 μM diltiazem. The time course of K-induced Mn uptake was biphasic: an initial faster phase and a following slower phase of accumulation. The extents of increments of the Mn contents were dependent on the order of the applications of K and Mn: the increments became smaller in the following order, 1) when Mn was applied prior to K, 2) Mn was applied simultaneously with K, 3) Mn was applied after K. These results suggested that superficially bound Mn penetrates into the smooth muscle cells of vas deferens during the stimulation by agonists through the voltage-dependent calcium-channel (VDC) and that intracellular Mn was hardly extruded. It was also suggested that the degree of activation of VDC, through which Mn can enter the cells, was in the following order, K > NE > ACh. These results were consistent with our previous report about the dual effects of Mn: the inhibition and potentiation of contractions. It was also suggested that Mn may be a useful tool as a Ca analogue because Mn can penetrate into cells through VDC and, once taken up into the cells, Mn is not readily extruded and remains in the cells even after extracellular Mn is washed away.

Keywords — manganese uptake; calcium-channel; norepinephrine; potassium; acetylcholine; guinea pig vas deferens

Introduction

Manganese (Mn) is known as an inorganic calcium antagonist and it inhibits the contractions of smooth muscles.1–6 However the accelerative effects of Mn on the contractions were also shown in some smooth muscles.6–8 In these reports it was presumed that intracellular Mn potentiated or induced tension developments, but the determination of Mn uptake to smooth muscle cells was not reported. Recently we have reported that Mn potentiated contractions caused by potassium (K) or norepinephrine (NE), but did not potentiate the acetylcholine (ACh)-induced contractions after repetitive application of Mn with these agonists in the guinea pig vas deferens.9–11 We suggested that the augmentation may be mediated by intracellular Mn accumulated with repetitive applications and that the amount of accumulated Mn may be different among the agonists applied with Mn. In the present study, we measured the Mn contents of the smooth muscle cells of guinea pig vas deferens under the various conditions using the atomic absorption spectrophotometry and characterized the Mn uptake. The results were discussed in relation to the dual effects of Mn, the inhibition and the potentiation, on the contractions.

Materials and Methods

Male guinea pigs of the Hartley strain weighing 350 to 550 g were sacrificed by a blow on the neck. The vasa deferentia were isolated and the connective tissues were removed. A strip in length of 8 to 10 mm was prepared from the middle portion of the vas deferens. The preparations were mounted under 0.3 g load in organ baths containing aerated HEPES-Locke-Ringer
solution (normal medium) of the following composition (mM): NaCl, 154; KCl, 5.6; MgCl₂, 2.1; CaCl₂, 2.2; glucose, 2.8; HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 4.4, adjusted to pH 7.4 at 30 °C with 1 N NaOH. CaCl₂ was omitted in the Ca-free medium and high-Ca medium contained 8.8 mM CaCl₂. Isotonic high-K medium was prepared by replacing 34.4 or 94.4 mM NaCl in the normal medium with equimolar concentration of KCl.

After the preparations were allowed to equilibrate in the normal medium for 90 min, isotonic contractions in response to 10 μM NE, 10 μM ACh and 100 mM K were recorded with 10 times magnification. Each of these agonists at these concentrations caused maximal phasic and nearly maximal (about 90% of maximal tonic contraction) tonic contractions. The magnitudes of the phasic and tonic phase of contractions induced by 40 mM K were about 60% of each maximal contractions. The application of K was carried out by substitution of the bathing medium with high-K medium. Only the preparations which exhibited reproducible contractions to the successive three times applications of agonists were used in the present experiments. The contraction to the 3rd application of an agonist was taken as the control contraction of the phasic and tonic components of the contractions, respectively. The contractile responses were expressed as the percentage of the control contraction of the same preparation. Diltiazem and Mn were applied for 10 min prior to and throughout the exposure to the agonists. Unless otherwise stated, agonists were applied for 5 min. For measurement of the time course of K-induced Mn uptake, K was applied for 1 to 15 min. In some experiments, Mn was applied simultaneously with or 5 min after the application of K. In both cases, the preparations were exposed to Mn for 5 min in the presence of K.

After appropriate treatments the preparations were washed 4 times with Ca-free medium. Each preparation was then blotted, weighed and dissolved in 1 ml HNO₃ for 72 h at room temperature. The sample was then diluted with distilled water to 10 ml, and the concentration of Mn was measured with atomic absorption spec-trophotometer (Hitachi, Type 170-10 or z-8000). The results were expressed as mean values ± S.E.M. Statistical analysis was made using the Student's paired t-test or unpaired t-test and was considered to be significant at the level of 5% probability.

The drugs used were: manganese chloride (Kanto Kagaku), l-norepinephrine (Nakarai), acetylcholine chloride (Daiichi-seiyaku), diltiazem (Tanabe).

Results

The Effect of Repetitive Applications of Manganese with Agonists on the Manganese Content of the Isolated Vas Deferens of Guinea Pig

When Mn was applied to the unstimulated vas deferens, a small amount of Mn was taken up and this Mn uptake was almost completed within 1 min. The repetitive applications of 2.1 mM Mn which showed a typical "tachyphylaxis" (9–11) did not increase the Mn contents of the unstimulated preparations (Fig. 1). After 7 times application, the Mn content was 0.86 ± 0.03 nmol/mg wet weight. In contrast, Mn contents were remarkably increased when 100 mM K was applied in the presence of Mn. Up to 7 times repetitive application of Mn with K, the

![Graph](image_url)

Fig. 1. The Effects of Potassium on Manganese Accumulation in Isolated Vas Deferens of the Guinea Pig

O, Mn (2.1 mM) applied without K; ●, Mn applied with K (100 mM). After 10 times treatments of Mn with or without K, the preparations were washed for 1 h in the normal medium with the application of K every 20 min (1' – 3'). Each point indicates means with S.E.M. indicated by vertical bars where they exceed the size of the symbol.
**Table I. The Effects of Agonists on the Manganese Uptake in Isolated Vas Deferens of the Guinea Pig**

<table>
<thead>
<tr>
<th>Concentration of Mn (mM)</th>
<th>Application times</th>
<th>Mn contents of preparations (nmol/mg wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.37</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.273 ± 0.013 (18)</td>
</tr>
<tr>
<td>K 100 mM</td>
<td>9</td>
<td>0.283 ± 0.010 (8)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.466 ± 0.018 (22)</td>
</tr>
<tr>
<td>NE 10 μM</td>
<td></td>
<td>0.692 ± 0.034 a(5)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.135 ± 0.050 a(8)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.684 ± 0.032 a(10)</td>
</tr>
<tr>
<td>ACh 10 μM</td>
<td></td>
<td>0.302 ± 0.004 (8)</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.382 ± 0.008 a(8)</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.564 ± 0.011 a(8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.544 ± 0.027 a(8)</td>
</tr>
</tbody>
</table>

The preparations were treated repeatedly with Mn with or without agonists (control) for 9 or 15 times. Values represent the mean ±S.E.M. a) Indicate that the values are statistically different from each control at \( p = 0.05 \) analyzed with Student's unpaired \( t \)-test. The number of preparations are shown in the parentheses.

**Table II. The Effects of Extracellular Calcium Concentrations on the Potassium-Induced Manganese Uptake in Isolated Vas Deferens of the Guinea Pig**

<table>
<thead>
<tr>
<th>Extracellular Ca (mM)</th>
<th>Mn contents (nmol/mg wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without K (a)</td>
</tr>
<tr>
<td>0</td>
<td>1.44 ± 0.03</td>
</tr>
<tr>
<td>2.2</td>
<td>0.86 ± 0.03</td>
</tr>
<tr>
<td>8.8</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

The preparations were treated with 2.1 mM Mn plus 100 mM K for 7 times. The values represent mean ±S.E.M. N.D., not determined. \( n \), the number of the preparations.

Mn content was increased linearly to 3.28 ± 0.08 nmol/mg wet weight and after 10 times repetitions the Mn content was 3.95 ± 0.17 nmol/mg wet weight. After 10 times repetitive application of Mn and K, K was applied without Mn for 3 times every 20 min, but the Mn content was not decreased (Fig. 1, dotted line).

When 2.1 mM Mn was applied with 40 mM K, the Mn content was also increased to 1.65 ± 0.07 nmol/mg wet weight after 10 times repetitions. This indicated that the Mn contents may be increased depending on the dose of agonists. However, the Mn uptake induced by agonists at the higher concentrations than that induced maximal contractions were not determined.

NE and ACh at 10 μM also significantly increased the Mn contents in the presence of 0.21 mM and 0.37 mM Mn except when using 0.21 mM Mn with ACh for 9 times (Table I). The degree of increase in Mn contents were dependent on the concentrations of Mn and were higher in NE-treated preparations than in ACh-treated preparations. Even after 15 times applications of Mn with ACh, the Mn contents were smaller than those after 9 times applications of Mn with NE. Further, when 0.37 mM Mn was applied with ACh, the Mn contents after 15 times applications were about the same as those after 9 times applications. The increase in the Mn contents induced by either agonist was remarkably smaller than those induced by K in the presence of the same concentrations of Mn.

**The Effect of Extracellular Calcium Concentration on the Uptake of Manganese**

Increasing the concentrations of extracellular Ca decreased the Mn content obtained without agonists (resting Mn uptake, Table II). The K-induced Mn uptake (the difference between the mean value of the Mn contents of the preparations applied Mn with K and the mean value of the resting Mn uptake) was smaller in the normal medium than in Ca-free medium. Further the K-induced Mn uptake in the normal medium was larger than that in high-Ca medium (Table II).

**The Effects of Diltilazem on the Uptake of**
TABLE III. The Effects of Diltiazem on the Phasic and Tonic Contractions Repeatedly Induced by Repetitive Applications of Potassium and Norepinephrine in Isolated Vas Deferens of Guinea Pig

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Phasic</th>
<th>Tonic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium (100 mM)</td>
<td>8</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>+ diltiazem (2.1 μM, 1st treatment)</td>
<td>8</td>
<td>93.7 ± 1.5</td>
<td>48.1 ± 2.9</td>
</tr>
<tr>
<td>+ diltiazem (2.1 μM, 9th treatment)</td>
<td>8</td>
<td>86.7 ± 2.1</td>
<td>24.4 ± 3.1</td>
</tr>
<tr>
<td>Norepinephrine (10 μM)</td>
<td>8</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>+ diltiazem (2.1 μM, 1st treatment)</td>
<td>8</td>
<td>96.4 ± 1.3</td>
<td>78.7 ± 3.8</td>
</tr>
<tr>
<td>+ diltiazem (2.1 μM, 9th treatment)</td>
<td>8</td>
<td>95.2 ± 1.0</td>
<td>73.4 ± 3.5</td>
</tr>
</tbody>
</table>

n, number of the preparations. The values represent mean ±S.E.M.

Manganese
Diltiazem (2.1 μM) inhibited both the phasic and tonic components of K- and NE-induced contractions significantly but the inhibitory effects were marked in the tonic components and stronger inhibition was observed in K-induced contractions than in NE-induced contractions (Table III). The same concentration of diltiazem decreased significantly the Mn contents of the preparations treated with 2.1 mM Mn plus K for 7 times (Table IV). The K-induced uptake of Mn was suppressed to 39% of that in the absence of diltiazem. Diltiazem also decreased the Mn contents of the preparations treated with 0.37 mM Mn and NE for 9 times but not the resting Mn uptake (Table V). The NE-induced Mn uptake was decreased by diltiazem to 30%.

The Effects of the Application Order of Manganese and Potassium on the Uptake of Manganese
The time course of K-induced Mn uptake was composed of two components: the faster

![Graph](https://via.placeholder.com/150)

Fig. 2. The Time Course of Potassium-Induced Manganese Uptake and the Inhibitory Effects of Diltiazem in Isolated Vas Deferens of the Guinea Pig

The preparations were treated with Mn (2.1 mM) for 4 times with or without K (100 mM). The values are obtained as differences between the mean Mn contents of the preparations treated with K and those without K. Open circle shows the Mn uptake in the presence of diltiazem (2.1 μM). The number of preparation was 8 for each point.
Fig. 3. The Effects of Application Order of Manganese and Potassium on the Rates of Manganese Uptake

The preparations were treated with Mn (2.1 mM) and K (100 mM) repeatedly. Closed circle and open circle show the Mn contents of the preparations applied Mn simultaneously with K and 5 min after K respectively. Each point represents mean. Standard errors were not indicated because they did not exceed the size of symbol (n = 8).

phase appeared during the initial 1 min depolarization and the slower phase appeared by further depolarization (Fig. 2). Both faster phase and slower phase were inhibited by 2.1 μM diltiazem to 45% and 30% respectively.

The rate of Mn uptake caused by repetitive applications of Mn and K was affected by the application order of Mn and K. When Mn was applied 10 min prior to the 5-min treatment with K in the presence of Mn, the rate of Mn uptake was greater than that caused by simultaneous application of Mn with K for 5 min (Fig. 1, 3). The rate of Mn uptake caused by the repetitive treatments of simultaneous application of Mn with K for 5 min was greater than that caused by the repetitive treatments of Mn in the presence of K which was applied 5 min prior to each application of Mn (Fig. 3).

Discussion

NE, ACh and K caused the accumulation of Mn in the guinea pig vas deferens. The Mn contents were increased according to the application times of Mn with agonists. Mn accumulated by 10 times repetition of the application of Mn with K were minimally extruded by 1 h washing. These results were compatible with the results obtained in myocardial cells of rat and frog.

There is a correlation between the increment of Mn contents and the degree of "tachyphy-
laxis". Both of them were dependent on the application times of Mn in the presence of agonists and the agonists used. ACh induced the smallest Mn uptake among the agonists. Consistent with this fact, the inhibitory effects of Mn on the ACh-induced contractions were hardly reversed by the repetitive applications of Mn. These results support the previous assumption that intracellular Mn which was accumulated with repetitive applications augmented the contractions in response to K and NE, and resulted in the progressive diminution of the inhibitory effects of Mn. In the previous reports, it was suggested that the augmenting effects of intracellular Mn on the contractions were not due to the direct action of Mn on the contractile proteins but due to the increasing availability of Ca. This assumption is also confirmed because Mn uptake was still observed in Ca-free medium while the augmenting effect on the contractions was hardly obtained in such a condition.

The K-induced Mn uptake was inhibited by the increase of extracellular Ca concentration and was enhanced in the Ca-free medium. Diltiazem also inhibited the K-induced uptake of Mn. These results suggest that Mn can penetrate into the cells through voltage-dependent Ca-channels (VDC) activated by K. Diltiazem also markedly inhibited the Mn uptake by NE. This indicated that NE-induced Mn uptake may be caused by the activation of VDC. However, the inhibitory effect of the same concentrations of diltiazem on the contraction induced by NE was rather weak. These results confirmed that the main Ca sources for the contraction induced by NE may be intracellular Ca stores and/or extracellular Ca penetrating into the cells through a receptor-operated Ca-channel (ROC). It is also suggested that the degree of the activation of VDC by ACh is smaller than that by NE.

The time course of K-induced uptake of Mn is biphasic. Drapeau et al. demonstrated the similar time course of voltage-dependent Mn uptake into isolated presynaptic nerve endings from rat brain. They concluded that Mn enters into the synaptosome via both 'fast' and 'slow' Ca channels such as Ca, Sr and Ba. There might be two or more kinds of Ca channels through which Mn penetrates into smooth
muscle cells of guinea pig vas deferens. Recently Ca channels were divided into three types. Of these, T-type and L-type channel have been demonstrated in various smooth muscles. In the present results, both faster and slower uptake of Mn were inhibited by diltiazem though the sensitivity of faster phase to diltiazem was lower than that of slower phase. Thus, both of these uptake phases appeared to be caused by the activation of L-type channels. Further investigations will be needed to characterize the different sensitivity of channels to diltiazem, through which Mn penetrates.

The rate of Mn uptake depended on the application order of Mn and K. The rate was fastest when Mn was applied 10 min prior to each K application, while it was lowest when Mn was applied during depolarization by K. This indicates that superficially bound Mn may preferentially enter into the cells. Although the exact mechanism of the Mn transport is not clear, a possible explanation is that Mn displaces Ca from the binding sites which closely couple to or locate on Ca channels.

The rate of uptake of Mn during the initial 5 min of K depolarization was faster than that during the later 5 min. This indicates that the Ca-channels are inactivated in a time dependent manner. However, the magnitude of the tonic component of the K-induced contraction was maintained at least for 10 min. It has been considered that the tonic component was maintained by a constantly elevated intracellular Ca concentration which is the consequence of an altered equilibration of Ca influx, Ca extrusion through the membrane, and Ca sequestration. If this hypothesis is correct and VDC is inactivated in a few minutes, the mechanisms of reduction of intracellular free Ca must be also inactivated. On the other hand, the mechanism of "latch" has been proposed to explain the sustained tonic contractions. Which mechanism is responsible for the maintenance of the tonic phase of K-induced contractions of the guinea pig vas deferens remains to be determined.

In conclusion, our results strongly suggest that Mn can enter into the guinea pig vas deferens through VDC and that intracellularly accumulated Mn may augment the contractions. Further it was demonstrated that Mn may be a useful tool as a Ca analogue to study Ca movements because, once Mn is taken up into cells through VDC activated by various agonists, intracellular Mn is not readily extruded and remains in the cells even after the elimination of extracellular Mn.

References


