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MANGANESE FLUXES AND MANGANESE-DEPENDENT NEUROTRANSMITTER RELEASE IN PRESYNAPTIC NERVE ENDINGS ISOLATED FROM RAT BRAIN

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SUMMARY

1. The uptake and efflux of ^{54}Mn and ^{45}Ca, and the release of dopamine (DA) were measured in pinched-off presynaptic nerve endings (synaptosomes) isolated from rat brain.

2. The uptake of Mn and Ca was increased when forebrain or striatal synaptosomes were incubated in a depolarizing, K-rich solution. The time courses of K-stimulated Mn and Ca entry were similar: there was initially a high rate of ion accumulation, lasting 1–3 s, that gradually levelled off. The initial uptake of Mn, like that of Ca, was greatly diminished by a 10 s pre-incubation in K-rich solution prior to the addition of radiotracer.

3. Several Ca channel blockers, including Ni (0·03 mM), Sr (2·0 mM), Co (0·04 mM), Ba (1·5 mM) and La (0·2 mM), suppressed the K-stimulated uptake of Mn and of Ca to a similar extent. The K-stimulated uptake of Mn increased as a function of the external Mn concentration, and saturated at high external concentrations of Mn. These high concentrations of Mn also blocked the K-stimulated uptake of Ca.

4. There was a decreased efflux of Ca, but not of Mn, from the synaptosomes when the external Na concentration was reduced. The Na-dependent efflux of Ca was diminished by external Mn, but was unaffected when the synaptosomes were loaded with Mn.

5. The rate of [^{3}H]DA release from striatal synaptosomes was less than 0·001 s^{-1} in non-depolarizing, low-K solutions, in the absence or presence of Mn and Ca (1 mM). The rate of release was also unchanged in depolarizing, K-rich solutions in the absence of these divalent cations. The addition of 1 mM-Mn to a K-rich solution increased the rate of DA release by about 40%, and the time course of release was linear for at least 30 s. The addition of 1 mM-Ca increased the rate of release nearly 100-fold during the first second, and thereafter the rate of release rapidly declined.

6. Ni (1 mM) and, to a lesser extent, Mg (10 mM) reduced the rate of K-stimulated DA release that is dependent on either Mn or Ca. The pattern of inhibition of DA release resembled the pattern of inhibition of K-stimulated uptake of Mn and Ca.

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7. The addition of Mn to K-rich solutions stimulated the release of the neurotransmitters 5-hydroxytryptamine and γ-aminobutyric acid, but not acetylcholine, from striatal synaptosomes. The addition of Ca stimulated the release of all three of these neurotransmitter substances. Neither Mn nor Ca increased the rate of release of glutamic acid.

8. These results indicate that Mn can compete with Ca for passage through the voltage-dependent Ca channels in synaptosomes. Mn can stimulate transmitter release from nerve terminals, but it is far less effective than Ca.

INTRODUCTION

Neurotransmitter release is dependent on the influx of Ca through ion-specific channels that are activated by the depolarization of presynaptic nerve terminals (Katz, 1969; Llinas, 1977). Sr and Ba can substitute for Ca in the release process, while a variety of transition metals suppress synaptic transmission and Ca influx (Ginsborg & Jenkinson, 1976). Mn, a first-series transition metal, has complicated effects on neurosecretion. Although it inhibits the nerve-evoked release of transmitter (Katz & Miledi, 1969; Meiri & Rahamimoff, 1972) and blocks many Ca channels (Hagiwara & Nakajima, 1966; Kerkut & Gardner, 1967), it can, in the absence of external Ca, increase the frequency of quantal release following tetanic nerve stimulation (Kita, Narita & Van der Kloot, 1981). In addition, Mn produces a long-lasting potentiation of acetylcholine (ACh) release from cardiac parasympathetic nerve endings (Bechem, Glitsch & Pott, 1981).

The relationship between Mn and transmitter release is of particular interest, because chronic exposure to this metal leads to symptoms in man resembling Parkinson's disease (Hornykiewicz, 1966). This may be due to Mn accumulation in the corpus striatum (Chandra, Srivastava & Shukla, 1979) and a concomitant decrease in dopamine (DA) levels in the same region (Mustafa & Chandra, 1971). Recently, Daniels, Gysling & Abarca (1981) demonstrated that striatal slices accumulated Mn, causing the release of DA. Thus, the neurological symptoms of Mn poisoning may be linked to the uptake of Mn by dopaminergic nerve terminals in the striatum.

In order to understand the relationship between Mn and neurosecretion better, we have measured Mn and Ca fluxes, and DA release, in isolated presynaptic nerve terminals (synaptosomes) prepared from rat brain. Synaptosomes retain many of the morphological and functional properties of intact neuronal tissue (Bradford, 1975; Blaustein, Kendrick, Fried & Ratzlaff, 1977). In particular, they maintain a resting membrane potential (Blaustein & Goldring, 1975), have voltage-activated Ca channels (Blaustein, 1975; Nachshen & Blaustein, 1980), and release DA in a voltage-and Ca-dependent manner (Drapeau & Blaustein, 1983).

Our experiments show that Mn can permeate the presynaptic Ca channels, and can also support the release of DA from depolarized nerve endings. Unlike Ca, however, Mn does not produce a phasic, initial burst of transmitter release. Mn is able to release neurotransmitters other than DA, suggesting that its effects on nerve terminals at short times of exposure are not limited to dopaminergic pathways in the C.N.S.

METHODS

Preparation of synaptosomes

The preparation of synaptosomes from rat forebrains has been described in previous publications (Krueger, Ratzlaff, Strichartz & Blaustein, 1979). In brief, a modification of the method of Hajos (1975) was employed. The P_2 pellet was resuspended in 0·32 M-sucrose, and layered onto 0·8 M-sucrose. After centrifugation at 10000 *g* for 30 min, the nerve terminal-enriched material in the 0·8 M-sucrose fraction was equilibrated by the gradual addition of 2–3 volumes of low-K solution containing (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 0·02; glucose, 10; HEPES, 10, adjusted to pH 7·5 at 3 °C with NaOH. The preparation of striatal synaptosomes was similar, except that the sucrose gradient step was omitted; the P_2 pellet was directly equilibrated as described above.

Measurement of radio-isotope influx and efflux

The equilibrated synaptosome or P_2 suspension was centrifuged (13000 *g*) for 10 min, and the pellet was resuspended in a low-K solution (pH 7·5 at 30 °C). The resuspended synaptosomes were gently agitated and warmed for 20 min at 30 °C before proceeding with the experiments.

Entry of radio-isotope was initiated by adding aliquots of the warmed suspension to equal volumes of low-K or K-rich solution with tracer (see Nachshen & Blaustein, 1980). Typically, each sample (400 μl) contained 0·5–1 μCi ^{45}Ca or ^{54}Mn (New England Nuclear). In the radiotracer flux experiments, choline solutions with a reduced Na concentration were used to determine the base-line accumulation of Mn and Ca, unless stated otherwise. These choline solutions contained (mM): NaCl, 72·5; choline Cl, 72·5; KCl, 5. In the corresponding K-rich solutions, the K concentration was increased by isosmotically substituting K for choline. Ca, Mn, and other ion concentrations are given for the specific experiments. In addition, all solutions contained glucose, Mg and pH buffers as indicated above.

Tracer uptake was terminated (quenched) by rapidly diluting the incubation media with 4·0 ml of ice-cold low-K solution containing 1–3 mM-$LaCl_3$. After quenching, the diluted synaptosomes were immediately filtered. The filters (Whatman, GF/A, or Schleicher and Schuell, no. 25) were then washed with two additional 4 ml aliquots of the quenching solution, and the radioactivity retained on the filters was determined with standard liquid scintillation counting techniques. The data were corrected for radio-isotope quenching and tracer binding to the filters. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Measurements were performed in quadruplicate, and are shown $\pm$ the standard error (S.E. of the mean), unless this fell within the data symbols.

In radio-isotope efflux experiments, the synaptosomes were loaded with radiotracer for 10 s by incubating them in a K-rich solution as described above. Efflux was initiated by diluting the suspensions 16-fold with tracer-free solutions; efflux was terminated by filtration as described above. The decrease in retained radioactivity compared to the initial load obtained in the K-rich solution was taken as a measure of efflux.

Measurement of neurotransmitter release

Equilibrated P_2 fractions were labelled with radioactive neurotransmitters by incubation for 30 min at 30 °C in low-K solution containing either: (i) 0·1 μM-[^{3}H]DA (100 Ci/mmol), 0·5 mM-ascorbate (to prevent oxidation) and 0·05 mM-pargyline (a monoamine oxidase inhibitor), (ii) 0·1 μM-[^{3}H]glutamate (100 Ci/mmol), (iii) 0·1 μM-[^{3}H]5-hydroxytryptamine (100 Ci/mmol), 0·5 mM-ascorbate and 0·05 mM-pargyline, (iv) 0·1 μM-[^{3}H]choline (200 Ci/mmol, to label the acetylcholine pools), or (v) 0·1 μM-[^{3}H] γ-aminobutyric acid (100 Ci/mmol) and 1 μM-aminooxyacetic acid (to prevent transamination). An aliquot of the labelled P_2 fraction (50 μl, containing 250 μg protein) was pipetted into one well of an Amicon VFM-2 filtration tank (Amicon Corp., Danners, MA) containing a glass-fibre filter, as described previously (Drapeau & Blaustein, 1983). The synaptosomes were washed free of unincorporated tracer by six repeated additions of low-K solution with no added Ca, followed by suction. The Ca concentration in solutions with no added Ca ranged from 5 to 15 μM, as determined by atomic absorption spectroscopy. Transmitter release from the synaptosomes was then initiated by adding either a low-K (5 mM-K and 145 mM-Na), choline (5 mM-K, 75 mM-Na and 70 mM-choline), or K-rich (75 mM-K and 75 mM-Na) solution, with glucose, Mg and pH buffer as indicated above. Some of the solutions also contained Ca or Mn, as specified for the individual

experiments. Release was terminated 1–30 s later by adding ice-cold low-K solution containing 20 mM-Ni (to block Ca uptake) and applying a vacuum. The collected effluent and the filter pads were assayed for radioactivity by liquid scintillation spectrometry. The results are expressed as the mean percentage of ^3H released (±S.E. of mean) based on the sum of the radioactivity remaining on the filter pads and in the samples collected; measurements were performed in quadruplicate.

TABLE 1. The uptake of Ca and Mn in forebrain and striatal synaptosomes

		Forebrain		Striatum	
Time (s)	Ion	145 mM-Na +5 mM-K	72·5 mM-Na +77·5 mM-K	145 mM-Na +5 mM-K	72·5 mM-Na +77·5 mM-K
1	Ca	0·075±0·005	0·241±0·001	0·054±0·002	0·149±0·006
10	Ca	0·234±0·018	0·769±0·03	0·146±0·007	0·443±0·019
1	Mn	0·025±0·001	0·044±0·001	0·025±0·002	0·039±0·002
10	Mn	0·065±0·003	0·147±0·007	0·056±0·003	0·096±0·005

Synaptosomes prepared from the forebrain or the striatum (see Methods) were incubated in solutions containing 0·02 mM-Ca as well as 0·01 mM-Mn, along with radiotracer (either ^{45}Ca or ^{54}Mn), for 1 or 10 s. The mean uptake values (nmol/mg protein) are shown ±S.E. of means ($n = 4$).

RESULTS

Mn and Ca uptake

When synaptosomes are incubated in a control, low-K solution containing 145 mM-Na and 5 mM-K, there is a time-dependent accumulation of ^{45}Ca (Blaustein, 1975; Nachshen & Blaustein, 1980) or of ^{54}Mn (Table 1). Uptake of both these divalent cations is substantially increased if half of the Na in the external solutions is replaced with K. These results are observed in both the forebrain and the striatal synaptosome preparations (Table 1). The magnitude of the Mn and Ca fluxes was smaller in the striatal preparation, probably because this preparation is less enriched in nerve terminals, and has more contaminant protein (e.g. from mitochondria). Since we were interested in studying the voltage- and Mn-dependent release of dopamine, most of our experiments utilized the striatal preparation that is enriched in dopaminergic nerve endings. In some of the flux studies, however, it was more convenient to utilize the forebrain preparation that yielded larger amounts of tissue, and gave larger flux values per milligram protein. As shown in Table 1, both preparations give qualitatively similar flux results: Replacement of Na with K increases the Ca uptake by a factor of ~3, while the Mn uptake is increased by a factor of two or less. In both preparations, the rate of influx declines substantially between one and ten seconds.

The extra uptake of Ca obtained when half of the Na is replaced by K has two components (Blaustein, 1975): most of the uptake is through voltage-dependent Ca channels that are activated by the K-induced depolarization; part of the uptake is through an exchanger (Na–Ca or Ca–Ca) that is activated by reduction of the external Na concentration (Fig. 1). Fig. 1 shows that the uptake of Mn in K-rich solution also has two components. There is a small increase in the Mn accumulation when half of the Na is replaced by choline; the accumulation is further increased if the synaptosomes are depolarized (Blaustein & Goldring, 1975) by replacing half of the Na with K instead of choline. These results suggest that Mn uptake in K-rich solution is mediated via the same mechanisms that mediate Ca uptake.

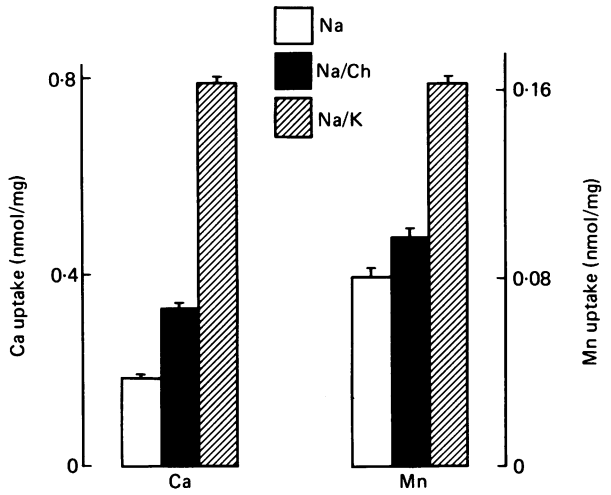

Fig. 1. The effect of replacing one-half of the external Na with choline or K on Ca and Mn uptake by striatal synaptosomes. Incubation with tracer (either ^{45}Ca or ^{54}Mn) lasted 10 s, in solutions containing 0·01 mM-Mn and 0·02 mM-Ca.

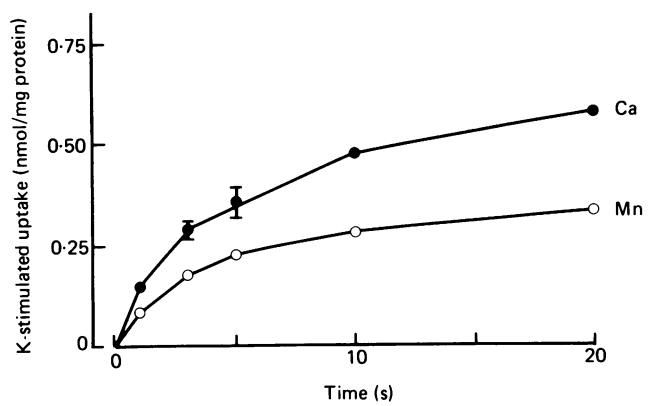

Fig. 2. The time course of K-stimulated Ca or Mn uptake in striatal synaptosomes. The solutions with radiotracer Ca (●) or Mn (○) contained 0·02 mM of both these divalent cations.

The idea that both Ca and Mn utilize the same Ca channels is supported by the similar time courses of entry for the two ions. Fig. 2 shows that the rate of voltage-dependent uptake (K-stimulated, i.e. uptake in K-rich solution minus the uptake in choline solution) of Mn and Ca is initially high, and gradually levels off. The similar time course of Mn and Ca influx is consistent with Mn entry via both 'fast' (inactivating) Ca channels, and 'slow' (non-activating) Ca channels, as has been previously reported for the voltage-dependent influx of Ca, Sr and Ba (Nachshen & Blaustein, 1980, 1982). It is possible to measure Ca influx through the slow channels by first inactivating the fast Ca channel population. This is done by pre-incubating the synaptosomes in a depolarizing solution prior to the addition of Ca (Nachshen & Blaustein, 1980). We compared the ability of Mn to pass through the fast and slow

Ca channels by measuring K-stimulated Mn uptake either during a 1 s incubation, or during a 5 s incubation after first inactivating the fast Ca channels by pre-depolarization. In one experiment with 0·02 mM-external Mn and Ca, the initial rate of K-stimulated Mn influx during a 1 s incubation declined 15-fold after pre-depolarization. Similar results were seen in another experiment with 0·5 mM-Mn in the incubating solutions. The initial rate of K-stimulated Ca influx in these experiments declined 3–5-fold. Thus Mn, like Ca, passes through both 'fast' (inactivating) and 'slow' (non-inactivating) Ca channels.

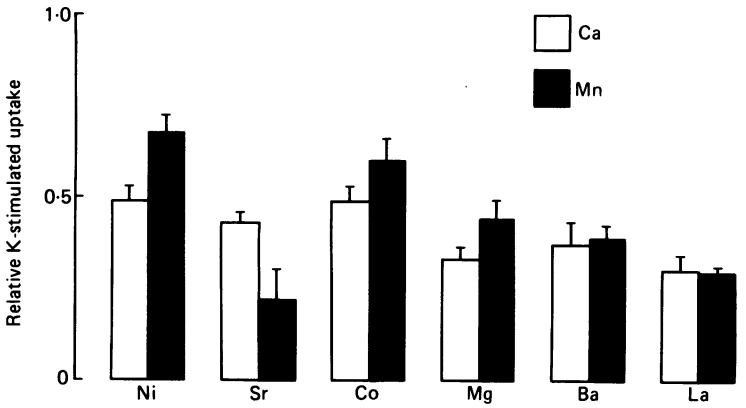

Fig. 3. The effect of Ca channel blockers on the K-stimulated uptake of Ca and Mn in striatal synaptosomes. Solutions with radiotracer ^{45}Ca (open bars) or ^{54}Mn (filled bars) contained 0·02 mM of both these divalent cations. The concentrations of the different blockers used were (mM): $NiCl_2$, 0·03; $SrCl_2$, 2·0; $CoCl_2$, 0·04; $MgCl_2$, 4·0; $BaCl_2$, 1·5; $LaCl_3$, 0·2. Incubation with radiotracers lasted 5 s. The results have been normalized to the K-stimulated uptake of Ca and Mn obtained in the absence of blockers.

To establish further the role of Ca channels in mediating Mn entry, we tested the effects of several Ca channel antagonists (Hagiwara & Byerly, 1981) on the K-stimulated influx of Ca and Mn, in double-tracer experiments (Fig. 3). Ni, Sr, Co, Mg, Ba and La all decreased the uptake of both divalent cations to a very similar extent, consistent with Mn entry via the voltage-dependent Ca channels.

Lee & Tsien (1983) have reported recently that the organic Ca antagonist methoxyverapamil (D600) is a better blocker of Ca current than of Ba current in Ca channels from guinea-pig ventricle cells. We therefore compared the effect of the related organic Ca antagonist, verapamil, on K-stimulated Mn and Ca influx. Verapamil was more effective in blocking the influx of Mn than of Ca (Fig. 4). This result was obtained both at low (0·02 mM; Fig. 4*A*) and high (1·0 mM; Fig. 4*B*) concentrations of Mn, with verapamil concentrations ranging from 10 to 80 μM, at incubation times from 1–10 s, and with both striatal and forebrain synaptosomes. One possible explanation for the preferential block of Mn influx is that verapamil modifies channel permeability for this ion in a selective way.

The effect of varying the external Mn concentration on the K-stimulated influx of both Ca and Mn was examined at incubation times ranging from 1 to 10 s. The results from one experiment, conducted at 5 s, are shown in Fig. 5*A*. At all times

tested, the Mn uptake increased as a function of increasing external Mn, and approached a maximal level (J_{max}) at high concentrations of Mn. Thus, the K-stimulated influx of Mn, like the K-stimulated influx of Ca, Sr and Ba (Nachshen & Blaustein, 1980, 1982) saturates with increasing permeant ion concentrations, as expected if Mn utilized the presynaptic Ca channels. Previous studies have demonstrated that Mn has a greater affinity for the 'fast' Ca channels than for the 'slow' Ca channels: during the first second of Ca uptake, the half-inhibition constant of Mn

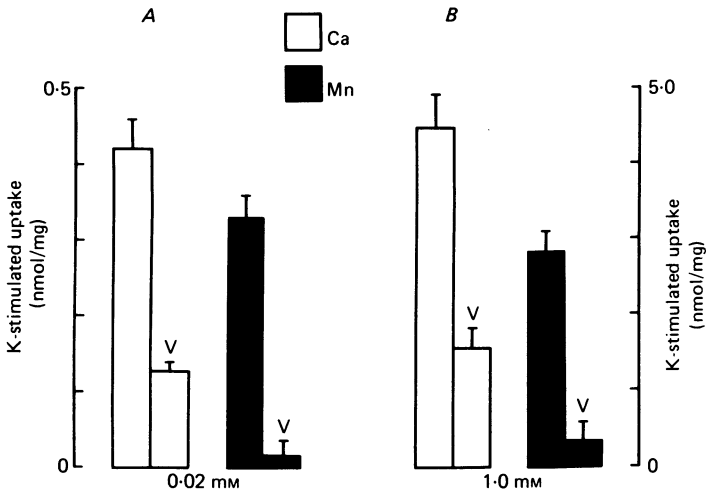

Fig. 4. The effect of verapamil (80 μM) on the K-stimulated uptake of Ca and Mn. Forebrain synaptosomes were added to choline- or K-rich solutions containing either ^{45}Ca (open bars) or ^{54}Mn (filled bars), and K-stimulated influx after 5 s was determined. The first bar of each pair shows the uptake in the absence of verapamil. The second bar shows the uptake in the presence of verapamil (V). *A*, the uptake was measured in the presence of either 0·02 mM-Ca or 0·02 mM-Mn. *B*, the uptake was measured in the presence of either 1·0 mM-Ca or 1·0 mM-Mn. All Mn-containing solutions also contained 0·02 mM (unlabelled) Ca.

($K_{I(Mn)}$) is approximately 70 μM, increasing to 300 μM by 10 s, after most of the 'fast' channels have inactivated (Nachshen & Blaustein, 1980). Accordingly, we found that the half-saturation constant (K_M) for K-stimulated Mn uptake was 30–50 μM at 1 s (not shown), and 100–200 μM at 5 s (Fig. 5*A*), further evidence that Mn passes through both the 'fast' and 'slow' Ca channels.

As the external Mn concentration was raised, there was a reduction in the K-stimulated Ca influx (open symbols, Fig. 5*A*). This also indicates that Mn and Ca were competing for the same channels (see Nachshen & Blaustein, 1982). We confirmed that Mn was a competitive inhibitor of Ca influx by measuring K-stimulated Ca influx at 1 s at several Ca concentrations, in the presence and absence of 60 μM-Mn. The results of this experiment were plotted in double-reciprocal form (Fig. 5*B*). The lines joining the data points extrapolated to a common intercept on the *Y*-axis, as expected for a competitive mode of inhibition. Furthermore, the calculated value of $K_{I(Mn)}$) in this experiment, 80 μM, was in the range of K_I and K_M values previously obtained for Mn at 1 s.

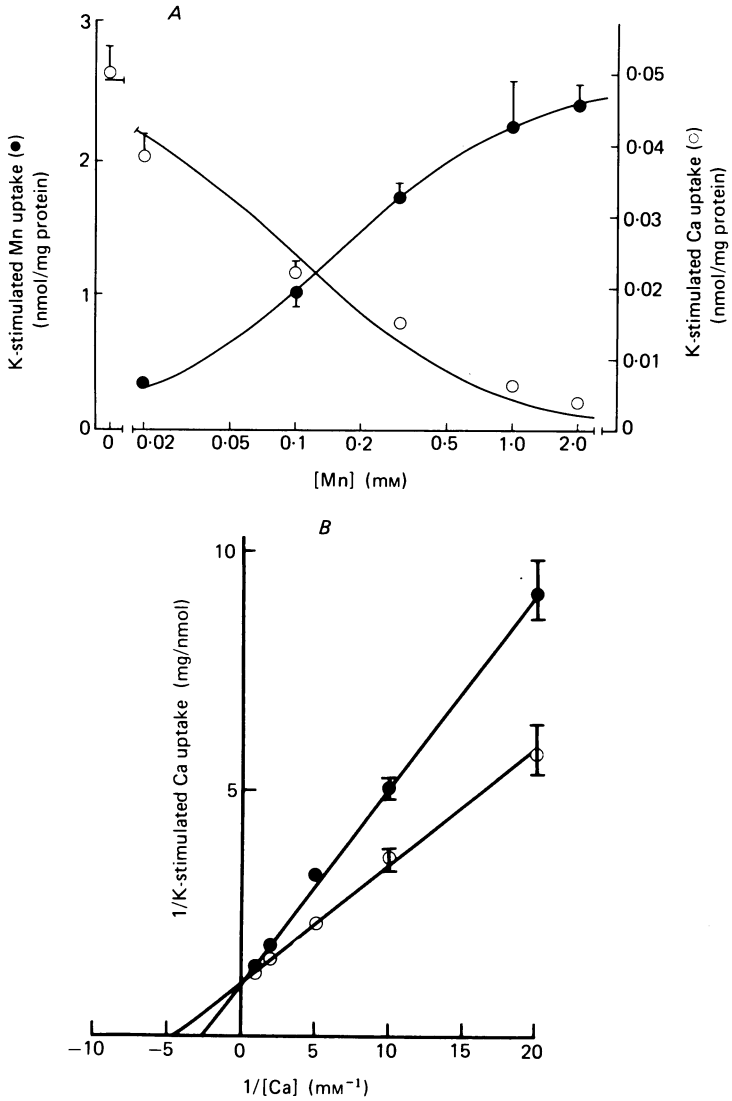

Fig. 5. *A*, the effect of increasing the Mn concentration on K-stimulated Ca (○) or Mn (●) uptake. All solutions contained 0·02 mM-Ca. Incubation lasted 5 s. Striatal synaptosomes were used in this experiment. The data points were fitted by the method of least squares to the equations (Nachshen & Blaustein, 1982): $J_{Mn} = J_{max(Mn)}/(1 + K_{M(Mn)}/Mn)$, ●; or $J_{Ca} = J^*_{Ca}/(1 + Mn/K_{I(Mn)})$, ○. J^*_{Ca} is the K-stimulated uptake of Ca in the absence of Mn at low Ca concentrations; other symbols used are explained in the text. The values derived from the curve-fitting were: $K_{I(Mn)} = 0{\cdot}10$ mM; $K_{M(Mn)} = 0{\cdot}15$ mM; $J_{max(Mn)} = 2{\cdot}6$ nmol/mg protein. *B*, the effect of Ca on K-stimulated Ca uptake in the presence (●) or absence (○) of 0·06 mM-Mn. Incubation lasted 1 s, with forebrain synaptosomes. The data are plotted in double reciprocal form. The value of $K_{M(Ca)}$, obtained by extrapolating the line joining the open symbols to the *X*-axis, was 0·22 mM. The $K_{I(Mn)}$ value obtained by extrapolating the line joining the filled symbols, was 0·08 mM after correcting for competition with Ca.

In a number of experiments the permeability of Ca relative to Mn (P_{Ca}/P_{Mn}) for the K-stimulated Ca channels was determined in solutions containing 0·02 mM-Ca and 0·01 mM- or 0·02 mM-Mn. The relative permeability is given by the following equation (Nachshen & Blaustein, 1982):

$$P_{Ca}/P_{Mn} = J_{Ca} \times [Mn]_0/J_{Mn} \times [Ca]_0, \quad (1)$$

where J_{Ca} and J_{Mn} indicate the K-stimulated influx of Ca and Mn, and $[Mn]_0$ and $[Ca]_0$ are these ions' external concentrations. The relative permeability was (mean and S.E. of mean) $2·5 \pm 1·1$ at 1 s ($n = 3$), $1·8 \pm 0·2$ at 5 s ($n = 3$) and $2·8 \pm 0·5$ at 10 s ($n = 4$). Thus, there was no indication of a significant change in relative channel permeability with time. In other experiments the relative permeability was similarly

TABLE 2. The Na dependence of Ca and Mn efflux

Efflux solution	T (°C)	Radiotracer	% loss (±S.E. of mean)
150 mM-choline Cl	3	Ca	6±4
150 mM-choline Cl	30	Ca	25±4
72·5 mM-NaCl, 77·5 mM-KCl	30	Ca	41±4
150 mM-choline Cl	3	Mn	1±6
150 mM-choline Cl	30	Mn	15±6
72·5 mM-NaCl, 77·5 mM-KCl	30	Mn	13±5

All efflux solutions contained 2 mM-EGTA, 5 mM-$MgCl_2$ and pH buffers, in addition to the ions shown in Table 1. Efflux was initiated by diluting the uptake solutions 16-fold with the efflux solutions, and efflux was terminated after 30 s by filtering and washing the samples, as described in the Methods. The synaptosomes were loaded with radiotracer during a 10 s incubation in K-rich solution containing both 0·05 mM-Ca and 0·1 mM-Mn, along with the appropriate radio-isotope. The uptake of ^{45}Ca (nmol/mg protein) was 1·15, and the uptake of ^{54}Mn was 1·18. The radiotracer loss during the 30 s efflux period is normalized to these values. This experiment was done using forebrain synaptosomes.

determined in solutions containing 1·0 mM-Mn and 0·02 mM-Ca, and was $3·5 \pm 1·5$ (pooled data from two experiments at 10 s, and one at 5 s); this result is not significantly different from results obtained at low Mn concentrations ($P > 0·05$, Student's t test), but because of the large error more experiments are needed to determine if Mn modifies the channel selectivity. The average P_{Ca}/P_{Mn} for all conditions ($n = 13$) was $2·6 \pm 0·4$.

Mn and Ca efflux

The efflux of Mn and Ca from synaptosomes was compared in a number of experiments, by loading the synaptosomes with similar amounts of either ^{45}Ca or ^{54}Mn, and measuring tracer retention after the synaptosomes were diluted into solutions without radio-isotope (Table 2). When the tracer-loaded synaptosomes were diluted into ice-cold solution (either 150 mM-choline, or 72·5 mM-choline and 77·5 mM-K (not shown)) without Na or Ca (+2 mM-EGTA), there was no significant loss of tracer after 30 s. There was some loss of Mn (15 %) and of Ca (25 %) after 30 s when the diluting solution was at 30 °C. When a solution containing 72·5 mM-Na and 77·5 mM-K was used to dilute the synaptosomes, Ca efflux increased to 41 %, but there was no further increase in the loss of Mn. Thus, as reported by Sanchez-Armass, Nachshen & Blaustein (1982), there is a Na-dependent efflux of Ca (Blaustein & Oborn, 1975; Blaustein & Ector, 1976), but not of Mn, from synaptosomes.

Blaustein & Ector (1976) had previously shown that external Mn was able to inhibit Ca efflux from synaptosomes. We therefore examined whether internal Mn could also inhibit the Na-dependent efflux of Ca. Synaptosomes were loaded by depolarization either in the presence of 0·02 mM-Ca, or in the presence of 1 mM-Mn and 0·1 mM-Ca. The external Ca concentration was adjusted to give similar Ca loads in the presence or absence of Mn, while the Mn uptake was four times greater than the Ca uptake. The synaptosomes were diluted into efflux solutions, and Ca efflux was determined. As shown in Table 3, internal Mn had no effect on the Na-dependent Ca efflux. When Mn was also present in the dilution solution, however, Na-dependent Ca efflux decreased by half. Similar results were seen in another experiment, in which the synaptosomes were loaded with either 0·05 mM-Ca or with 0·3 mM-Mn and 0·1 mM-Ca.

TABLE 3. The effect of internal or external Mn on Na-dependent Ca efflux

	Efflux solution	T (°C)	% loss (±S.E. of mean)
A	150 mM-choline Cl	3	3±4
	72·5 mM-NaCl, 77·5 mM-KCl	30	41±4
B	150 mM-choline Cl	3	5±10
	72·5 mM-NaCl, 77·5 mM-KCl	30	45±7
	150 mM-choline Cl, 1·1 mM-$MnCl_2$	3	4±13
	72·5 mM-NaCl, 77·5 mM-KCl, 1·1 mM-$MnCl_2$	30	25±7

All efflux solutions contained 0·1 mM-EGTA, 1 mM-$MgCl_2$ and pH buffers in addition to the ions shown in Table 2. Efflux was initiated and terminated as described in the legend to Table 1 and in the Methods. Efflux lasted 30 s. The uptake of radio-isotope lasted for 10 s in K-rich solution with ^{45}Ca containing either 0·02 mM-Ca (A), or 0·1 mM-Ca and 1·0 mM-Mn (B). The uptake of ^{45}Ca (nmol/mg protein) was 1·48 in A and 2·10 in B. In separate determinations using ^{54}Mn, Mn uptake in B was found to be 8·56 nmol/mg protein. Forebrain synaptosomes were used in this experiment.

Mn and neurotransmitter release

The effect of changing the external Na and K concentrations on [^{3}H]DA release from striatal synaptosomes, in the presence or absence of Mn and Ca, is shown in Fig. 6. When the synaptosomes were incubated in a control, low-K solution, 3·4 % of the total label was released in 30 s. The addition of 1 mM-Ca or -Mn to this solution did not cause any significant change in release. Replacement of half the Na by choline also had very little effect on DA release. In some experiments a small, but significant, increase in DA release was noted when Ca was added to the choline-containing solution; however, no significant effect of Mn addition to the choline solution was noted in any of these experiments. When the synaptosomes were depolarized by replacement of half the Na with K instead of with choline, DA release remained nearly at its base-line level in the absence of Ca or Mn, but was stimulated in the presence of either of these divalent cations. The small increase in release observed in low-Na solutions containing Ca is probably due to the increase in Ca uptake observed under similar conditions (Fig. 1). Failure to observe an increase in DA release in low-Na solutions containing Mn suggests that this ion is less effective in promoting neurosecretion. In seventeen experiments, the addition of 1 mM-Mn to a K-rich solution increased release over a 30 s period by 39±7 % (mean and S.E. of mean), with the

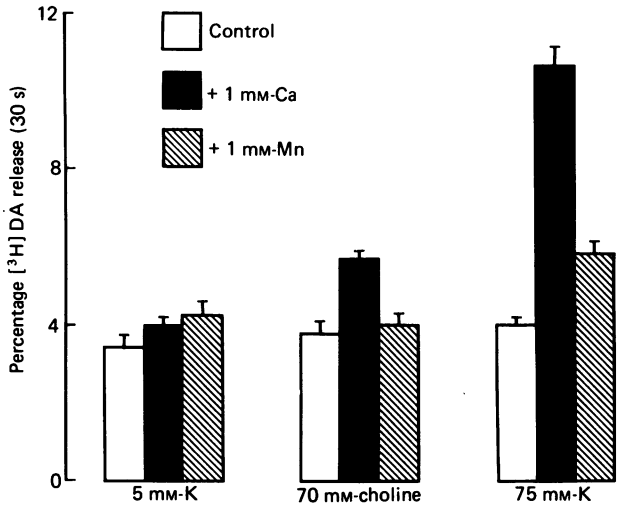

Fig. 6. The effect of replacing one-half of the external Na with choline or K on DA release, in the presence or absence of Ca and Mn. [^{3}H]DA release was measured 30 s after the addition of either low-K, choline or K-rich solutions to the synaptosomes. The control solutions (open bars) contained no Mn or added Ca. Other solutions contained either 1 mM-Ca (filled bars) or 1 mM-Mn (hatched bars).

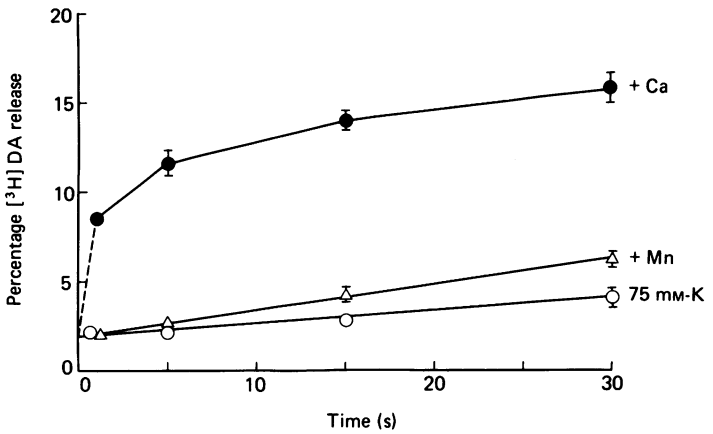

Fig. 7. The time course of Ca- and Mn-dependent DA release. Release was initiated by adding K-rich solutions to the filters at time zero. These solutions contained either 1 mM-Ca (●), 1 mM-Mn (△) or no Mn or added Ca (○).

stimulation ranging from no effect (seven experiments) to a 2·1-fold increase (one experiment). The effect of adding 1 mM-Ca to a K-rich solution was consistently larger, with an average increase over a 30 s period of $250 \pm 10\%$ (seventeen experiments). These results strongly suggest that the increased [^{3}H]DA release is due to the entry of Mn or Ca into the synaptosomes that are depolarized by the K-rich solutions.

Fig. 7 shows that when synaptosomes loaded with [^{3}H]DA were incubated in a Ca-

and Mn-free K-rich solution, 2 % of the [^{3}H]DA was collected during the initial second. This presumably represents DA released during the 20 s required to change solutions, since it is also observed in changing from one control, low-K solution to another (Drapeau & Blaustein, 1983). During the subsequent 29 s, release continued at a low, constant rate of 0·0007 s^{-1}. When 1 mM-Ca was present in the K-rich solution, the rate of DA release during the initial second (corrected for the initial wash-out) increased nearly 100-fold (rate constant = 0·067 s^{-1}), and then greatly decreased over the following 29 s. The time course of DA release parallels Ca entry through the

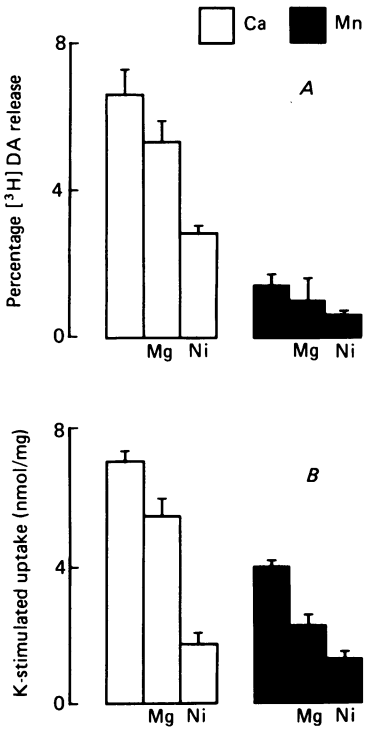

Fig. 8. *A*, the effect of Mg (10 mM) and Ni (1 mM) on Ca- and Mn-dependent release of DA. Transmitter release was measured 30 s after the addition of K-rich solutions containing either 1 mM-Ca (open bars) or 1 mM-Mn (filled bars). The values shown have been corrected for DA release obtained in the corresponding K-rich solutions (±Ni and Mg) with no Mn or added Ca. *B*, the effect of Mg (10 mM) and Ni (1 mM) on the K-stimulated uptake of Ca and Mn in forebrain synaptosomes. Uptake was measured at 30 s, in solutions containing either 1 mM-Ca (open bars) or 1 mM-Mn (filled bars). The uptake values in choline solution without Ni or Mg were (nmol/mg protein) 6·9±0·1 for Ca, and 4·5±0·1 for Mn.

inactivating and non-activating Ca channels described above (and see Nachshen & Blaustein, 1980), as shown in an earlier study (Drapeau & Blaustein, 1983). Though the time courses of K-dependent Ca and Mn uptake were similar (Fig. 2), the time courses of DA release in the presence of these two divalent cations were quite different. Thus the time course of DA release in the presence of 1 mM-Mn was linear (rate constant = 0·0015 s^{-1}) and, after correcting for the initial wash-out, extrapolated to the origin with no evidence of a high initial rate.

We examined the effects of several Ca channel antagonists on K-stimulated DA release mediated either via Mn or Ca. Ni (1 mM) and Mg (10 mM) reduced both the Ca- and Mn-dependent release (Fig. 8*A*). The pattern of inhibition of DA release resembled the pattern of inhibition of K-stimulated ^{45}Ca or ^{54}Mn influx, measured under similar conditions (Fig. 8*B*). This is consistent with the notion that the Mn-mediated release of DA depends on the entry of Mn into the nerve endings through the presynaptic Ca channels. Results with the Ca channel blockers verapamil (0·1 mM) and Cd (1 mM) were inconclusive, because these agents increased the levels of base-line DA release, making measurement of Mn-dependent release difficult.

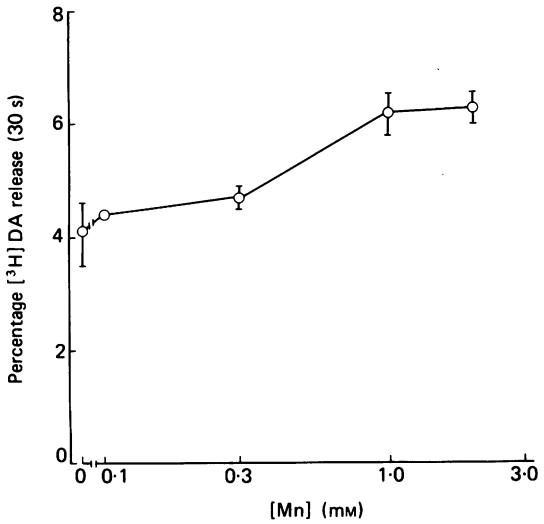

Fig. 9. The effect of varying the Mn concentration on DA release. [^{3}H]DA release was measured 30 s after the addition of K-rich solutions with the indicated concentrations of Mn to the synaptosomes. These solutions contained no added Ca.

The K-stimulated release of [^{3}H]DA measured at 30 s increased as the external concentration of Mn was raised above 0·1 mM, and saturated at a Mn concentration of 1 mM (Fig. 9). Because of the relatively small amount of Mn-dependent DA release, the concentration of Mn producing half-maximal release could not be determined accurately, but was between 0·3 and 1 mM.

Although both Ca and Mn increased the rate of transmitter release, their effects were not additive. The K-stimulated release of [^{3}H]DA was measured with sub-maximal concentrations of either Ca or Mn, or with both of these divalent ions present at the same time (Fig. 10). When both ions were added to the K-rich solution, the DA release was always intermediate to the release obtained with either divalent ion alone, i.e. the action of Ca and Mn was not synergistic. This also suggests that Ca and Mn ions are not acting on separate pools of labelled DA or on separate populations of synaptosomes.

We examined the effects of Mn and Ca on the K-stimulated release of several striatal neurotransmitters other than DA. In the absence of Ca and Mn, similar rates of DA, 5-hydroxytryptamine (5-HT) and ACh, and somewhat higher rates of glutamic acid (Glu) and γ-aminobutyric acid (GABA) release, were observed (Fig. 11).

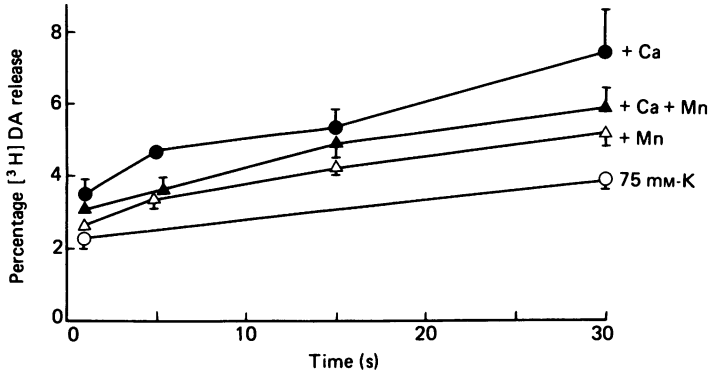

Fig. 10. The effect of Mn together with Ca on DA release. Release measurements were made at the times indicated, in K-rich solutions containing (mM): Mn, 0·3 (△); Ca, 0·1 (●); Mn, 0·3 and Ca, 0·1 (▲); no Mn or added Ca (○).

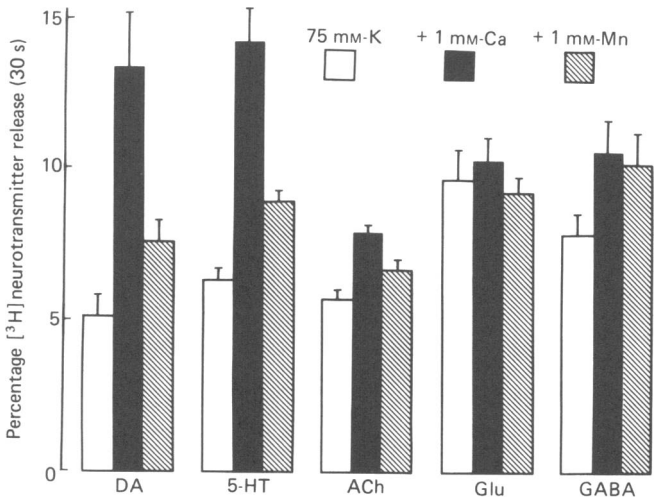

Fig. 11. The Ca and Mn dependence of DA, 5-HT, ACh, Glu and GABA release from striatal synaptosomes. Release of the tritiated neurotransmitters was measured 30 s after the addition of K-rich solutions with 1 mM-Ca (filled bars), 1 mM-Mn (hatched bars), or no Mn or added Ca (open bars).

The addition of Ca to the K-rich solution increased the rate of DA and 5-HT, and to a lesser extent, ACh and GABA release; K-stimulated release of Glu was unaffected by Ca. Mn also increased the K-stimulated release of DA, 5-HT and GABA, although to a lesser extent than Ca; the release of ACh and Glu was not significantly increased, perhaps because our assay was not sufficiently sensitive.

DISCUSSION

Mn fluxes in nerve terminals

Mn is generally considered to be a blocker of the voltage-dependent Ca channels at the presynaptic nerve terminal (Katz & Miledi, 1969; Meiri & Rahamimoff, 1972). Our results show, however, that in addition to its blocking effects, Mn can also permeate through brain presynaptic Ca channels. There is evidence that Mn passes through the Ca channels in starfish egg cells (Hagiwara & Miyazaki, 1977), insect larvae muscle (Fukuda & Kawa, 1977), guinea-pig papillary muscle (Ochi, 1975), and polychaete myoepithelial cells (Anderson, 1979). The Ca channels in many other types of cells are, however, impermeable to Mn (e.g. Hagiwara & Nakajima, 1966; Kerkut & Gardner, 1967). Thus the Ca channel, unlike the Na channel, has very dissimilar ionic selectivities in different preparations (for review see Edwards, 1982).

It has been proposed that Ca channel permeability can be characterized by two factors (see Hagiwara & Byerly, 1981): one factor is the rate of ion association with a binding site in the channel, and this is reflected in the half-saturation constant (K_M) of the permeating ion; the other factor is the rate of ion mobility through the channel after the binding step has occurred, and this is reflected in the maximal rate of ion influx (J_{max}) at saturating concentrations of the permeant ion. If this model is correct, the relative permeability of two competing ions, X and Y, can be calculated as (Nachshen & Blaustein, 1982):

$$P_X/P_Y = (J_{max(X)} \times K_{M(Y)})/(J_{max(Y)} \times K_{M(X)}), \quad (2)$$

where P_X/P_Y is the relative permeability. We used this equation to calculate the permeability of Ca relative to Mn after 1 s of depolarization in forebrain synaptosomes, from experimentally derived parameters: $K_{M(Mn)} = 70\ \mu M$ and $J_{max(Mn)} = 0{\cdot}24$ nmol/mg protein . s; $K_{M(Ca)} = 320\ \mu M$ and $J_{max(Ca)} = 2{\cdot}7$ nmol/mg protein . s (Nachshen & Blaustein, 1982). The calculated value, 2·5, is in reasonable agreement with the value of 2·6 obtained by measuring the relative permeability directly in double-label experiments. Thus, a simple model, involving the binding of permeating divalent cations to a site in the channel, can explain the concentration dependence of Mn influx, as well as the concentration dependence of Ca, Sr and Ba influx in synaptosomes (Nachshen & Blaustein, 1982). Since the half-saturation constant of Mn is smaller than that of Ca, Mn may bind to the Ca channel more easily. Once in the channel, however, it has a lower mobility, as evidenced by its much smaller maximal influx rate. Both the higher affinity and lower mobility of Mn may be due to its greater charge density (it has an ionic radius of 0·97 Å, compared to 1·14 Å for Ca), and its tendency, as a transition metal, to polarize electron donor groups. These factors might favour interactions with co-ordinating groups within the channel.

We observed a Na-inhibited influx of both Ca and Mn in synaptosomes. However, only Ca efflux, and not Mn efflux, was Na-dependent. It has been suggested that Na-dependent Ca fluxes in synaptosomes are mediated via a Na–Ca exchanger, similar to that found in many other preparations (Blaustein & Osborn, 1975; Blaustein & Ector, 1976). One possible explanation of our results is that the influx and efflux of Ca are mediated by way of separate pathways, with the latter being

unable to transport Mn. Alternatively, Mn may be more readily sequestered than Ca after it enters the nerve terminal (Rasgado-Flores, Nachshen & Blaustein, 1982), so that its cytosolic concentration is too low for significant exchange with external Na.

Blaustein & Ector (1976) have reported that external Mn blocks both the Na- and Ca-dependent efflux of Ca from synaptosomes; a similar block is seen in barnacle muscle fibres (Russell & Blaustein, 1974). Na-dependent Ca efflux from synaptosomes was not, however, diminished by loading the nerve terminals with Mn. As we suggest above, it may be that internal Mn does not interact with the exchange mechanism, or that the internal Mn concentration is not high enough to block Ca extrusion.

Possible mechanisms of Mn-induced neurotransmitter release

The addition of Mn to control, low-K solution did not cause any release of DA from striatal synaptosomes. After the synaptosomes were depolarized by high-K solution, however, the addition of Mn (1 mM) increased DA release by approximately 40 % over a 30 s period. Since depolarization in the absence of Mn (or Ca) produced no release. These results indicate that transmitter release is dependent on the Mn influx induced by high K. The observation that the Ca channel antagonists Ni and Mg reduced both Mn influx and Mn-dependent release of DA is consistent with this idea.

How does intraterminal Mn induce the release of neurotransmitter? Two possibilities are likely: Mn could either substitute for Ca in the exocytotic process, or it could induce the release of Ca from intraterminal stores. If Mn does act directly on the release mechanism, it does not activate exocytosis as rapidly as Ca, since the time course of release seen with Mn is far slower and more linear than that seen with Ca. Mn may be a less effective activator than Ca, or it may be present at lower cytosolic concentration, because of greater intraterminal buffering (Rasgado-Flores *et al.* 1982). Another possibility is that separate mechanisms or release sites control the initial phasic component and the prolonged slow component of neurotransmitter release: Ca may activate both mechanisms, while Mn activates only the slow component.

Our finding that the Ca efflux from synaptosomes is not significantly altered by internal Mn may argue against the displacement of Ca from its intraterminal stores. However, since the effect of Mn on Ca extrusion has not been examined directly, i.e. under conditions where the cytoplasmic Ca and Mn composition can be controlled, we cannot exclude an effect of Mn on stored Ca.

For brief depolarizations of the nerve terminal, as might be produced by a nerve action potential, our data suggest that Mn influx would not elicit any significant release of neurotransmitter. However, an increase in transmitter release might be detected after prolonged or repeated nerve stimulation, and this has been observed at the frog neuromuscular junction by Kita *et al.* (1981). These researchers found an increase in the frequency of miniature end-plate potentials in the absence of Ca and in the presence of Mn, after either tetanic stimulation or depolarization in high-K solution. Similarly, Bechem *et al.* (1981) measured a late-developing potentiation of transmitter release from guinea-pig cardiac parasympathetic nerve terminals after repetitive nerve stimulation in the presence of Mn. Their results led both groups of workers to hypothesize that Mn might be entering the nerve terminals through voltage-activated channels, and we confirm that this does indeed occur in nerve terminals isolated from rat brain.

Mn-dependent release of 5-HT and of GABA, as well as of DA, was detected in striatal synaptosomes. Thus, Mn is non-specific in its effects on evoked neurotransmitter release from nerve endings. These results suggest that the short-term effects of Mn may not be related to the Mn intoxication syndrome (and DA depletion) that is seen after chronic exposure to this metal. It is possible, however, that dopaminergic nerve terminals are more susceptible than are other nerve terminals to the long-term effects of Mn, or that they can chronically accumulate larger Mn loads.

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