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THE INHIBITORY EFFECT OF Mn^{2+} ON THE ATP-DEPENDENT Ca^{2+} PUMP IN RAT BRAIN SYNAPTIC PLASMA MEMBRANE VESICLES

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Abstract—Synaptic plasma membrane (SPM) vesicles take up Ca^{2+} when both ATP and Mg^{2+} are added to the reaction medium. Maximal ATP-dependent Ca^{2+} uptake is obtained with between 3 and 5 mM Mg^{2+}. Higher [Mg^{2+}] results in a slight decrease in the ATP-dependent Ca^{2+} influx, which at 10 mM Mg^{2+} is equal to 70% of the maximal uptake. [Mn^{2+}] up to 0.3 mM, can support ATP-dependent Ca^{2+} uptake as efficiently as equimolar [Mg^{2+}]. Maximal ATP-dependent Ca^{2+} uptake in the presence of Mn^{2+} is obtained between 1 and 2 mM but its extent is only 70% of the maximal uptake obtained in the presence of Mg^{2+}. As the [Mn^{2+}] increases the ATP-dependent Ca^{2+} uptake decreases and at 10 mM Mn^{2+} it is only 40% of that obtained in the presence of Mg^{2+}. The reduction in ATP-dependent Ca^{2+} uptake in the presence of Mn^{2+} is obtained also when Mg^{2+} is present in the reaction medium. The effect is independent of the [Mg^{2+}] used and depends on the [Mn^{2+}] alone. Studying the relationship between [Mn^{2+}] and [Ca^{2+}] revealed three patterns: at 0.3 mM Mn^{2+} this ion could replace Mg^{2+} over the entire [Ca^{2+}] range tested, 5–200 μM, supporting ATP-dependent Ca^{2+} uptake. When the [Mn^{2+}] was increased to 1.8 mM, it exhibited a competitive behaviour with Ca^{2+} which resulted in an increase in apparent K_m to Ca^{2+} of the SPM Ca^{2+} pump from 10.69 μM (SD = 3.49) to 28.88 μM (SD = 21.08). Four millimolar Mn^{2+} inhibited ATP-dependent Ca^{2+} uptake to 50% of that obtained in equimolar Mg^{2+} over the entire [Ca^{2+}], 5–200 μM, tested. No transport of $^{54}Mn^{2+}$ by the SPM Ca^{2+} pump could be detected. Inclusion of Mn^{2+} in the reaction medium led to excessive phosphorylation of SPM proteins. The excessive levels of phosphorylation persisted also when Ca^{2+} and/or Mg^{2+} were present in the reaction media.

Manganese toxicity is a real industrial and environmental hazard manifested especially in the lung and the nervous system [1, 2], but its mechanism of action is unknown. In its chronic form, manganese neurotoxicity resembles symptoms obtained in Parkinson's disease [3, 4] although no evidence has been obtained to show that dopamine pathways are primary targets. Other suggestions to explain manganese neurotoxicity involve glutathione metabolism [5], decrease in tissue levels of thiols [6] and increased production of reactive oxygen species [7].

To evaluate the exact toxic concentrations of Mn^{2+} is complicated. No relation was found between blood levels of Mn^{2+} and toxic symptoms [2]. This is a result of the very short half-life of Mn^{2+} in blood (1.28 min) and its accumulation in tissues [2].

Ca^{2+} ions have a major role in the function of the nervous system and there are several lines of evidence linking the action of Mn^{2+} with Ca^{2+} metabolism of nerves. $^{54}Mn^{2+}$ was shown to enter nerve terminals via voltage-dependent Ca^{2+} channels [8] in a competitive manner with Ca^{2+} and to inhibit evoked Ca^{2+}-dependent transmitter liberation [9], but also to substitute partially for Ca^{2+} in dopamine release from synaptosomes [8]. Mn^{2+} enters brain mitochondria via the Ca^{2+} uniporter [5] and accumulates there. It inhibits both Na^+-dependent and Na^+-independent Ca^{2+} efflux pathways [10]. The non-mitochondrial, endoplasmic reticulum type Ca^{2+} uptake system is also inhibited by Mn^{2+} [11] as well as the Na^+–Ca^{2+} exchanger [12, 13].

In the intact squid giant axon and bullfrog ventricles Mn^{2+} affected the ATP-dependent Ca^{2+} uptake system with low affinity [13, 14]. But since these studies involved intact preparations to which Mn^{2+} ions were added from the outside, its exact concentration within the cell was not known. Moreover, the Ca^{2+} pump activity was estimated by indirect means since both the Ca^{2+} and ATP binding sites are not accessible from the extracellular side. Since the synaptic plasma membrane (SPM†) Ca^{2+} pump is of major importance in the regulation of intracellular Ca^{2+} ion concentration and since Mn^{2+} was shown to affect neurotransmitter liberation we re-examined the effects of Mn^{2+} on the ATP-dependent Ca^{2+} pump directly. To do so, we used a preparation enriched in synaptic plasma membranes [15–19]. This membrane vesicle preparation takes up Ca^{2+} when ATP and Mg^{2+} are added to the extravesicular medium indicating that some of the vesicles are oriented with their cytoplasmic face to the outside (inside-out vesicles) and hence accessible to externally added Mn^{2+}, Ca^{2+} and ATP.

MATERIALS AND METHODS

Synaptic plasma membranes were prepared from

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† Abbreviations: SPM(s), synaptic plasma membranes; SDS, sodium dodecyl sulfate; EGTA, 2,2′-ethylenedioxybis[ethyliminodi(acetic acid)].

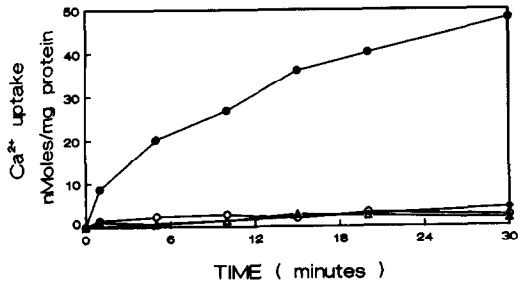

Fig. 1. ATP-dependent Ca^{2+} uptake in rat brain SPMs. SPMs (about 15 μg protein) were preloaded with 0.2 M KCl and 0.01 M Tris–HCl (pH 7.5) as described in Materials and Methods. Three microlitres of these vesicles were diluted into 100 μL 0.2 M KCl, 0.01 M Tris–HCl (pH 7.5), 100 μM $^{45}CaCl_2$ (0.1 μCi/100 μL) with 2 mM ATP and 5 mM $MgCl_2$ (●) or without ATP (○) or without $MgCl_2$ (△) or with 5 μM A23187 (◇). The reactions were carried out at 23° and stopped at the times indicated. The amount of $^{45}Ca^{2+}$ associated with the vesicles was determined as described in Materials and Methods. The experiment was repeated three times with this preparation of SPMs; and was routinely carried out with each new preparation of membranes used.

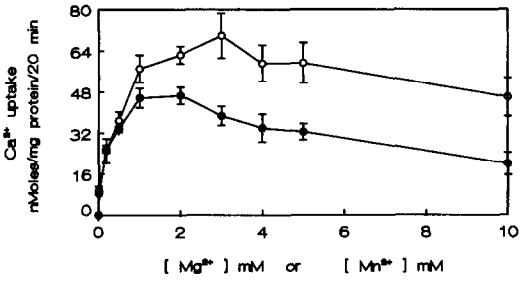

Fig. 2. The effect of different $[Mg^{2+}]$ and $[Mn^{2+}]$ on the ATP-dependent Ca^{2+} uptake in SPM vesicles. SPM vesicles (3 μL, 10–15 μg) preloaded with 0.2 M KCl and 0.01 M Tris–HCl (pH 7.5) were diluted into 100 μL of 0.2 M KCl, 0.01 M Tris–HCl (pH 7.5), 100 μM $^{45}Ca^{2+}$ (0.1 μCi/100 μL), 2 mM ATP and either 0–10 mM $MgCl_2$ (○) or 0–10 mM $MnCl_2$ (●). The reactions were stopped after 20 min of uptake at 23° and the Ca^{2+} associated with the vesicles was determined. The amount of Ca^{2+} associated with the vesicles in the absence of ATP at each $[Mg^{2+}]$ and $[Mn^{2+}]$ was subtracted. It never exceeded 10% of the amount of Ca^{2+} taken up by the vesicles in the presence of ATP. The data presented in this figure were obtained from 4 different experiments. Data points were determined from duplicate or triplicate measurements. The bars represent SEM.

osmotically shocked synaptosomes obtained from 14-day-old rat brains as described in detail previously [15–18]. The preparation has been characterized [15, 16] by measuring different marker enzyme and transport activities and found to be highly enriched in synaptic plasma membranes.

To measure ATP-dependent Ca^{2+} transport the membranes were pre-equilibrated by incubation with a 40-fold volume excess of 0.2 M KCl solution containing 0.01 M Tris–HCl buffer, pH 7.5, at 37° for 20 min. At the end of the incubation the membrane vesicles were concentrated by centrifugation for 20 min at 27,000 *g* for 4° and suspended in a small aliquot of the same solution which was used for pre-equilibration. Ca^{2+} transport was initiated by diluting 3–5 μL of the vesicles (about 10–20 μg protein) into 100 μL of external solution which was composed of 0.2 M KCl buffered with 0.01 M Tris–HCl, pH 7.5, and contained also 2 mM ATP, $^{45}CaCl_2$, Mg^{2+} and Mn^{2+} as specified. The amount of $^{45}Ca^{2+}$ associated with the vesicles in the absence of ATP and zero-time controls were determined as well. The reactions were stopped by dilution with 2 mL ice-cold 0.2 M KCl and the vesicles were collected by filtration using 0.45 μm Schleicher and Shuell nitrocellulose filters. The filters were washed two more times with the same KCl solution, dried and counted using a liquid scintillation counter. Each experiment was performed at least four times and the data points were calculated from duplicate or triplicate measurements.

ATP hydrolysis was determined by measuring the amount of $^{32}P_i$ liberated from [γ-^{32}P]ATP as described [19].

SDS–polyacrylamide gel electrophoresis was carried out using the method of Laemmli [20].

Phosphorylation of SPMs was determined by incubating the KCl preloaded membranes with 2 μM [γ-^{32}P]ATP (5000 mCi/mmol) and additions as specified in the legends of the appropriate figures for 20 sec at 23°. The reactions were stopped by adding SDS to a final concentration of 2%. The phosphorylated membranes were separated from unhydrolysed ATP by gel filtration using Sephadex G50 mini columns which were partially "dried" by a brief centrifugation using a bench top clinical centrifuge [21]. Prior to gel electrophoresis protein was determined by the method of Lowry *et al.* [22]. To permit comparison identical amounts of protein were loaded into each well.

$^{45}Ca^{2+}$, [γ-^{32}P]ATP and $^{54}Mn^{2+}$ were purchased from Amersham (Amersham, U.K.). Biochemicals were purchased from Sigma (Israel). All the reagents used were of analytical grade.

RESULTS

Synaptic plasma membrane vesicles were pre-loaded in a buffered KCl containing solution (see Materials and Methods) and diluted into a solution of identical composition, except that it contained ATP, Mg^{2+} and $^{45}Ca^{2+}$ as well. Figure 1 shows the Ca^{2+} accumulation with time by these vesicles. It can be seen that the Ca^{2+} uptake is ATP- and Mg^{2+}-dependent since in the absence of ATP and/or Mg^{2+} very small amounts of Ca^{2+} are taken up by the vesicles. Moreover, addition of the Ca^{2+} ionophore A23187 to the uptake medium in the presence of ATP and Mg^{2+} prevents Ca^{2+} accumulation.

Manganese ions can in part substitute for Mg^{2+} in supporting ATP-dependent Ca^{2+} uptake. Figure 2 compares the Mg^{2+} and Mn^{2+} concentration dependence of the ATP-dependent Ca^{2+} uptake. Experimental conditions are identical to Fig. 1, except that the Mg^{2+} or Mn^{2+} concentrations are varied. It can be seen that Mg^{2+} activates the system

in a concentration dependent fashion up to about 3 mM. Increasing the Mg^{2+} concentration further leads to a small decrease in the steady state level of Ca^{2+} taken up by the vesicles. At 10 mM Mg^{2+} the ATP-dependent Ca^{2+} uptake is about 70% of that obtained at 3 mM. Substitution of Mg^{2+} with Mn^{2+} also exhibits a biphasic behaviour. Low Mn^{2+} concentrations, up to 0.5 mM, can replace Mg^{2+} and support, as efficiently, ATP-dependent Ca^{2+} transport in SPM vesicles. Increasing [Mn^{2+}] up to 1 mM leads to an increase in the ATP-dependent Ca^{2+} uptake, but the amount of Ca^{2+} taken up by the vesicles is lower as compared to the uptake induced by identical [Mg^{2+}]. Increasing the Mn^{2+} concentration in the medium above 2 mM leads to a decrease in ATP-dependent Ca^{2+} influx. At 10 mM Mn^{2+} only about 40% of the amount of Ca^{2+} is taken up by the vesicles as compared to the maximal uptake at 1–2 mM Mn^{2+} and only about 40% of the amount taken up by 10 mM Mg^{2+}.

Several experiments were done in an attempt to understand the mechanistic basis of the reduction in the ATP-dependent Ca^{2+} transport in synaptic membranes when Mg^{2+} is replaced by Mn^{2+}.

First, the relationship between [Mg^{2+}] and [Mn^{2+}] was examined when present together in the vesicles external medium. Synaptic plasma membrane vesicles were preloaded in the same buffered KCl solution as in Fig. 1 and diluted into an external medium of identical composition to which ATP, Mg^{2+} and Mn^{2+} were added together. Appropriate controls without ATP and with ATP in the presence of only one of the divalent cations, Mn^{2+} or Mg^{2+}, were also done. In the experiment shown in Fig. 3A, saturating amounts of Ca^{2+} (100 μM), ATP and Mg^{2+} (5 mM) were used and the Mn^{2+} concentration was varied between 0 and 5 mM. In Fig. 3B, the same amounts of Ca^{2+} and ATP were used, the [Mn^{2+}] was 5 mM and the [Mg^{2+}] was varied between 0 and 5 mM. The Ca^{2+} taken up in the presence of ATP and either Mn^{2+} (Fig. 3A) or Mg^{2+} (Fig. 3B) alone is shown as well. The Ca^{2+} associated with the vesicles in the absence of added ATP in each case is also shown. It can be seen (Fig. 3A) that addition of low [Mn^{2+}] such as 0.5 mM to the 5 mM Mg^{2+}-containing reaction medium reduced the ATP-dependent Ca^{2+} uptake somewhat. Increasing the Mn^{2+} concentration to 1 mM, led to 40% reduction in the ATP-dependent Ca^{2+} uptake which did not change significantly till 5 mM Mn^{2+} was reached. The reduction in ATP-dependent Ca^{2+} uptake obtained when Mn^{2+} is added to the Mg^{2+}-containing media does not seem to represent an additive effect of [Mn^{2+} + Mg^{2+}], since comparable levels of Mg^{2+} alone (6–10 mM) maintained 70–90% of the steady state level of ATP-dependent Ca^{2+} uptake (see Fig. 2). Moreover, addition of Mn^{2+} alone (Fig. 3A, filled circles) led to similar low levels of ATP-dependent Ca^{2+} uptake between 1 and 5 mM as that which was obtained in the combined presence of Mg^{2+} and Mn^{2+}. The independence of the inhibitory action of Mn^{2+} from the Mg^{2+} concentration present in the reaction medium is shown also in Fig. 3B, where Mn^{2+} concentration was kept constant at 5 mM and the Mg^{2+} concentration was varied. Thus, it seems, that Mn^{2+} exerts its inhibitory action on the ATP-dependent Ca^{2+} transport in SPM vesicles by a "Mg^{2+}-independent pathway".

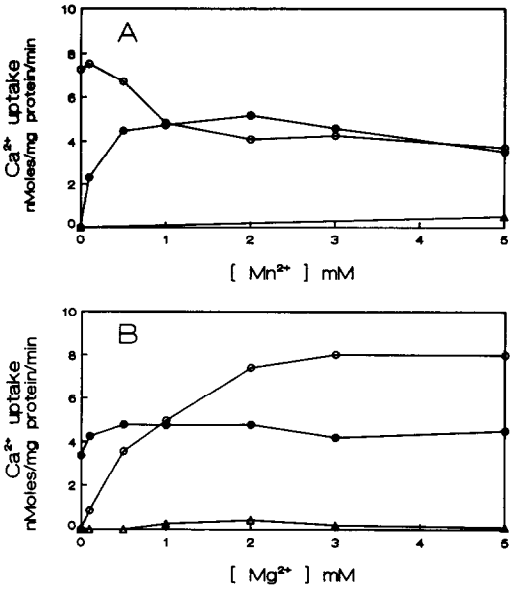

Fig. 3. The relationship between [Mg^{2+}] and [Mn^{2+}] in supporting ATP-dependent Ca^{2+} uptake. (A) SPMs (3 μL, 10 μg protein) preloaded with 0.2 M KCl and 0.01 M Tris–HCl (pH 7.5) were diluted into 100 μL of 0.2 M KCl, 0.01 M Tris–HCl (pH 7.5), 100 μM $^{45}CaCl_2$ (0.1 μCi/100 μL), 2 mM ATP, varying concentrations of Mn^{2+} with 5 mM $MgCl_2$ (○) or without (●). The Ca^{2+} associated with the vesicles in the absence of ATP (△) is also shown. The Ca^{2+} uptake was determined after 1 min at 23°. (B) Three microlitres of the same SPMs were diluted into 100 μL of 0.2 M KCl, 0.01 M Tris–HCl (pH 7.5), 100 μM $^{45}CaCl_2$ (0.1 μCi/100 μL), 2 mM ATP, varying concentrations of Mg^{2+} with 5 mM $MnCl_2$ (●) or without (○). The Ca^{2+} associated with the vesicles in the absence of ATP is also shown (△). The experiment was repeated four times.

The second type of experiment examined the relationship between [Ca^{2+}] and the extent of Mn^{2+} inhibition. In Fig. 4A the [Ca^{2+}] dependence of the ATP-dependent Ca^{2+} uptake into SPM vesicles at 0.3 mM Mn^{2+} and at 0.3 mM Mg^{2+} was examined, in Fig. 4B the ATP-dependent Ca^{2+} uptake at 1.8 mM Mn^{2+} and 1.8 mM Mg^{2+} was compared and in Fig. 4C the Ca^{2+} concentration dependence of the inhibitory action of 4 mM Mn^{2+} was tested. These Mn^{2+} concentrations were chosen since at saturating 100 μM Ca^{2+} (see Fig. 2), 0.3 mM Mn^{2+} supported ATP-dependent Ca^{2+} uptake as efficiently as 0.3 mM Mg^{2+}; in 2 mM Mn^{2+} the highest ATP-dependent Ca^{2+} uptake that this ion could support was obtained and at 4–5 mM Mn^{2+} about 50% inhibition of the ATP-dependent Ca^{2+} uptake as compared to similar [Mg^{2+}] was consistently obtained. It can be seen that at 0.3 mM (Fig. 4A) and 4 mM (Fig. 4C) Mn^{2+} or Mg^{2+}, the relationship between the ATP-dependent Ca^{2+} uptake obtained in Mn^{2+} relative to that obtained in identical [Mg^{2+}] is independent of the [Ca^{2+}] used. When 0.3 mM Mn^{2+} exchanges with 0.3 mM Mg^{2+}, no inhibition of the ATP-dependent Ca^{2+} uptake is obtained

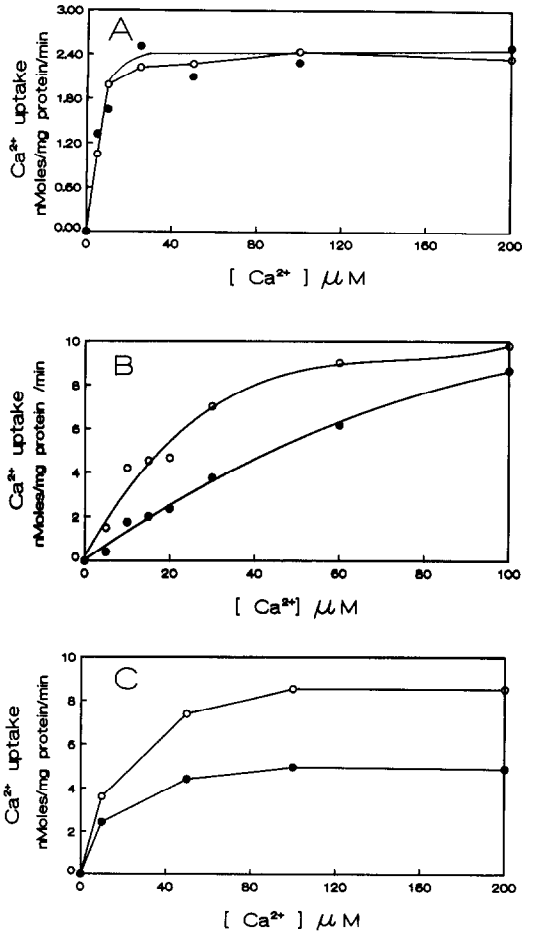

Fig. 4. The effect of different [Ca^{2+}] on the inhibitory effect of Mn^{2+} on the ATP-dependent Ca^{2+} uptake in SPM vesicles. (A) SPM vesicles (3 μL, about 10 μg protein) preloaded with 0.2 M KCl and 0.01 M Tris–HCl (pH 7.5) were diluted into 100 μL of an identical medium that contained also 2 mM ATP and either 0.3 mM $MgCl_2$ (○) or 0.3 mM $MnCl_2$ (●) and 0–200 μM $^{45}Ca^{2+}$. The $^{45}Ca^{2+}$ taken up by the vesicles in the absence of ATP was determined and subtracted. The reactions were carried out for 1 min at 23° and terminated as described in Materials and Methods. (B) Experiment identical to Fig. 4A except that the [Mg^{2+}] and [Mn^{2+}] concentrations were 1.8 mM. (C) Experiment identical to Fig. 4A and B except that the [Mg^{2+}] and [Mn^{2+}] concentrations were 4 mM. The experiment was repeated six times.

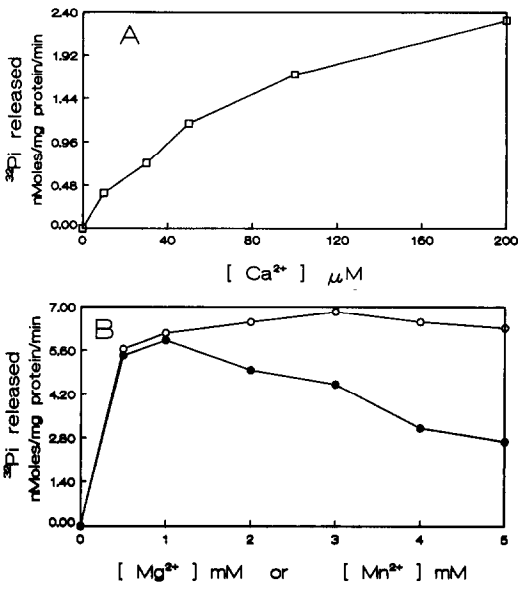

Fig. 5. The effect of Ca^{2+}, Mg^{2+} and Mn^{2+} on ATP hydrolysis. SPM vesicles (3 μL, about 10 μg protein) preloaded with 0.2 M KCl and 0.01 M Tris–HCl (pH 7.5) were diluted into 100 μL of reaction medium of identical composition, except that it contained also 2 mM [γ-^{32}P] ATP (5000 cpm/nmol) and either: (A) 0–200 μM $CaCl_2$; or (B) 0–5 mM Mg^{2+} (○) or Mn^{2+} (●) and 10 μM $CaCl_2$. The reactions were terminated after 1 min as described in Materials and Methods and the amount of $^{32}P_i$ liberated was determined. The experiment was repeated three times.

between 5 and 200 μM [Ca^{2+}] (Fig. 4A). When 4 mM Mn^{2+} substitutes 4 mM Mg^{2+}, the ATP-dependent Ca^{2+} uptake is about 50% inhibited throughout the entire [Ca^{2+}] range tested (Fig. 4C). When, however, 1.8 mM Mn^{2+} was used, increasing the [Ca^{2+}] decreased the inhibition obtained by Mn^{2+} (Fig. 4B). A Lineweaver–Burk plot of the relationship between the ATP-dependent Ca^{2+} uptake in the presence of Mn^{2+} or Mg^{2+} at different [Ca^{2+}] (not shown) indicated that the inhibitory action of Mn^{2+} was competitive with Ca^{2+}. The apparent K_m to Ca^{2+} in the presence of Mn^{2+} increased almost 3-fold. Calculating the average apparent K_m to Ca^{2+} from four different experiments similar to that shown in Fig. 4B indicated that it corresponded to 10.69 μM (SD = 3.49) in the presence of Mg^{2+} and to 28.88 μM (SD = 21.08) in the presence of Mn^{2+}. No change in the apparent V_{max} was observed which was 8.63 (SD = 1.82) nmole Ca^{2+}/mg protein/min in the presence of Mg^{2+} and 8.002 (SD = 2.78) in the presence of Mn^{2+}.

To find out whether Mn^{2+} competition with Ca^{2+} involved only binding, or transport as well, a similar experiment to that shown in Fig. 2 was carried out except that $^{54}Mn^{2+}$ was used and the Ca^{2+} was unlabeled. No $^{54}Mn^{2+}$ transport could be detected (not shown).

Figure 5 shows that Mn^{2+} is able to support ATP hydrolysis. The extent of ATP hydrolysis obtained in the presence of Mn^{2+} is compared to that obtained in the presence of Ca^{2+} and Mg^{2+}. In Fig. 5A the [Ca^{2+}] dependence of ATP hydrolysis is shown, in Fig. 5B the dependence of ATP hydrolysis on the presence of [Ca^{2+} + Mg^{2+}] and [Ca^{2+} + Mn^{2+}] are shown. We could not determine the effect of Mn^{2+} on the Ca^{2+}-dependent component of the ATP hydrolysed in the presence of Mg^{2+} and Ca^{2+} since this value can be deduced only in the presence of EGTA which eliminates all residual Ca^{2+} [19]. EGTA binds to Mn^{2+} with a higher binding constant than to Ca^{2+} [24]. The stability constants are 10.9 for Ca^{2+}, 5.4 for Mg^{2+} and 12.3 for Mn^{2+}. Addition of EGTA as Ca^{2+} chelator in the presence of Mn^{2+} reduces considerably free [Mn^{2+}] levels. It can

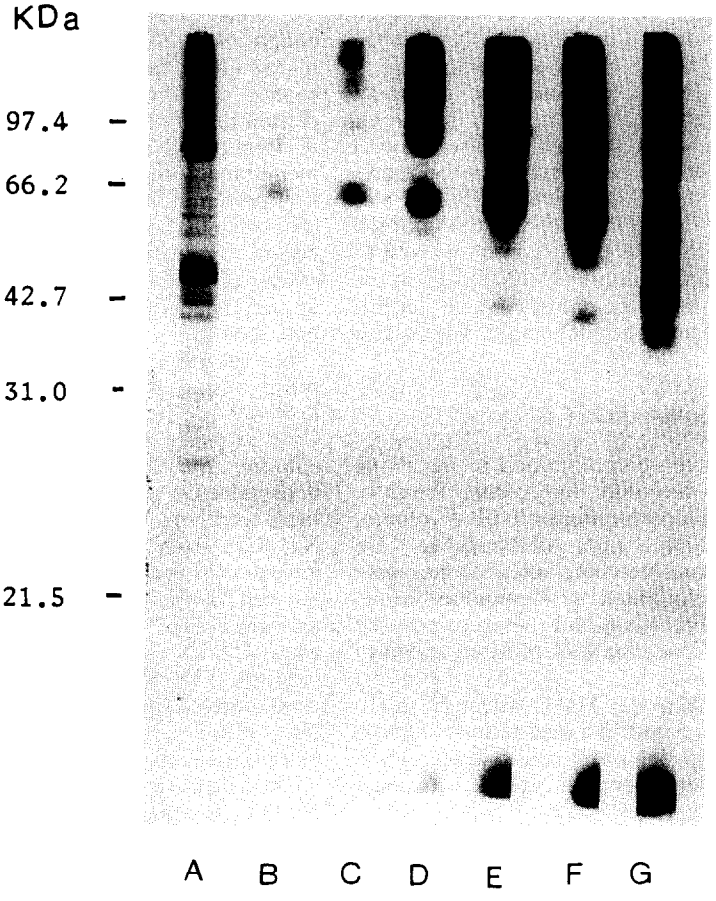

Fig. 6. Phosphorylation of SPM proteins. SPMs, preloaded with 0.2 M KCl and 0.01 M Tris–HCl, pH 7.5 (about 50 μg/lane) were incubated in 50 μL of a solution of identical composition except that it contained also 2 μM [γ-^{32}P]ATP (5000 Ci/mmol) alone (lane B) or with: 100 μM Ca^{2+} (lane C); with 500 μM Mg^{2+} and 100 μM Ca^{2+} (lane D); 20 μM Mn^{2+} (lane E); 20 μM Mn^{2+}, 100 μM Ca^{2+} and 500 μM Mg^{2+} (lane F); 50 μM Mn^{2+} (lane G). In lane A the Coomassie Blue stained protein profile of SPM proteins is shown. Each lane contains identical amounts of protein. Reactions were stopped after 20 sec by the addition of SDS to a final 2%; phosphorylated protein was separated from non-hydrolysed [γ-^{32}P]ATP by gel filtration (see Materials and Methods).

be seen that the hydrolysis of ATP obtained in the presence of Ca^{2+} alone is the lowest (Fig. 5A). Inclusion of Mg^{2+} or Mn^{2+} in the reaction medium leads to an increase in the ATP-dependent Ca^{2+} uptake. [Mg^{2+}] alone between 1 and 5 mM maintains the steady state level of ATP hydrolysis. Increasing [Mn^{2+}] over 1 mM leads to a concentration-dependent decrease in ATP hydrolysis (Fig. 5B) which is comparable to the effects of Mn^{2+} on the ATP-dependent Ca^{2+} uptake.

ATP-dependent Ca^{2+} uptake involves a phosphorylation step as part of the Ca^{2+} translocation cycle. In addition, Ca^{2+}- and calmodulin-dependent phosphorylation was shown to regulate the SPM Ca^{2+} pump [19]. Therefore, it was of interest to examine whether any change in the phosphorylation pattern of SPM proteins could be detected when Mn^{2+} exchanged Mg^{2+} and/or Ca^{2+}. Figure 6 shows an autoradiogram of SPMs preloaded with buffered KCl (see Materials and Methods) and incubated with 2 μM [γ-^{32}P]ATP. Membrane proteins were separated by SDS–polyacrylamide gel electrophoresis after removal of non-hydrolysed ATP by gel filtration. In lane A, a Coomassie Blue stained protein profile of the SPM proteins is shown. Since the amount of membrane protein loaded into each well of the SDS–polyacrylamide gel was identical, all the stained protein profiles are identical to that shown in lane A. It can be seen that very little phosphorylation is obtained when ATP alone is added to the membranes (lane B). Addition of Ca^{2+} alone at a concentration of 100 μM supports some phosphorylation of SPM proteins (lane C). Addition of Mg^{2+} at a concentration of 500 μM to the Ca^{2+} containing medium, increases somewhat the extent of membrane protein phosphorylation (lane D). Addition of 20 μM Mn^{2+} to the membranes alone (lane E) or in the presence of both Ca^{2+} and Mg^{2+} (lane F) results in a considerable increase in membrane protein phosphorylation. This phosphorylation seems to be of similar extent in all the 20 μM Mn^{2+}

containing reaction mixtures and independent from the presence of either $[Ca^{2+}]$ or $[Mg^{2+}]$ or both. Increase in $[Mn^{2+}]$ to 50 μM without any increase in either $[Ca^{2+}]$ or $[Mg^{2+}]$ increases the extent of phosphorylation further as can be seen in lane G. Higher $[Mn^{2+}]$ such as 100 or 500 μM (not shown) increased the extent of phosphorylation even more. Experiments (not shown) in which [α-^{32}P]ATP was used instead of [γ-^{32}P]ATP, to answer the question of whether this extensive phosphorylation pattern resulted from binding of non-hydrolysed ATP to the membranes in the presence of Mn^{2+}, ruled out this possibility since no phosphorylation of SPM proteins was obtained.

DISCUSSION

In this work we have investigated in detail the effect of Mn^{2+} on the SPM Ca^{2+} pump. We have used a preparation highly enriched in SPM vesicles, that take up Ca^{2+} when both ATP and Mg^{2+} are added to their external medium, indicating that some of these vesicles are oriented "inside-out" respective to the intact nerve terminal and therefore exposed directly to known concentrations of Mn^{2+} added to the reaction medium.

At saturating $[Ca^{2+}]$, Mn^{2+} affected ATP-dependent Ca^{2+} transport in a dual fashion: up to a concentration of 0.5 mM Mn^{2+} could replace Mg^{2+} and support ATP-dependent Ca^{2+} uptake with equal efficiency; increasing $[Mn^{2+}]$ above 0.5 mM led to a concentration-dependent reduction in Ca^{2+} transport as compared to similar $[Mg^{2+}]$. Between 3 and 5 mM Mg^{2+}, when optimal ATP-dependent Ca^{2+} uptake is attained, its substitution with Mn^{2+} resulted in a reduction of about 50% in the amount of Ca^{2+} taken up by the vesicles. Since Mn^{2+} could, in part, replace Mg^{2+} in supporting ATP-dependent Ca^{2+} transport, it was of interest to try and understand the mechanistic basis of its inhibitory action.

ATP-dependent Ca^{2+} translocation across the plasma membrane can be resolved into several partial reactions [23]. Some of these are Mg^{2+}-dependent, while others are Ca^{2+}-dependent. Interference of Mn^{2+} in some or all of these could result in an overall reduction in ATP-dependent Ca^{2+} uptake by the vesicles.

To examine the relationship between Mn^{2+} and Mg^{2+} in supporting ATP-dependent Ca^{2+} translocation, both ions were added together. These experiments indicated that the reduction in Ca^{2+} transport was independent of the $[Mg^{2+}]$ in the medium and occurred whether Mg^{2+} was present or not. This finding suggests that the inhibitory action of Mn^{2+} does not involve simple competition for binding sites with Mg^{2+}.

The relationship between Mn^{2+} and $[Ca^{2+}]$ was investigated at three different $[Mn^{2+}]$. At 1.8 mM Mn^{2+} and $[Ca^{2+}]$ between 5 and 200 μM, Mn^{2+} inhibited ATP-dependent Ca^{2+} transport in a competitive manner with Ca^{2+}. It did not change the apparent maximal reaction velocity but increased the apparent K_m to Ca^{2+} about 3-fold. No competition between Mn^{2+} and Ca^{2+} could be detected at 0.3 or 4 mM Mn^{2+}, presumably since the first requires $[Ca^{2+}]$ below 5 μM and the second above 0.5 mM. To reduce $[Ca^{2+}]$ below 5 μM, EGTA has to be added. Since, however, EGTA binds Mn^{2+} with higher affinity than it binds Ca^{2+} [24], the experiment cannot be carried out. In the millimolar Ca^{2+} concentration range, on the other hand, substantial ATP-independent Ca^{2+} binding to the synaptic membranes occurs, masking the amount of Ca^{2+} transported in an ATP-dependent manner. Thus, it is possible that competition between Ca^{2+} and Mn^{2+} occurs over a wide concentration range, and the extent of inhibition of the ATP-dependent Ca^{2+} uptake demands on the relative concentrations of both ions.

For sustaining normal physiological activity, the resting intracellular $[Ca^{2+}]$ in the nerve terminal is kept very low [25] by the combined action of several Ca^{2+} extrusion and sequestration pathways. Mn^{2+} entering the nerve terminal through voltage-dependent Ca^{2+} channels [8, 9] is expected to stay there for a long time since it is not extruded by the Na^+–Ca^{2+} exchanger [14] or by the Ca^{2+} pump, as indicated in the experiments using $^{54}Mn^{2+}$. Inside the cell, it inhibits mitochondrial Ca^{2+} transport pathways [10], Na^+ gradient-dependent Ca^{2+} pathways [12, 14] and also, as our results clearly indicate, ATP-dependent Ca^{2+} extrusion pathways. Consequently, it affects Ca^{2+} homeostasis which in turn would affect all Ca^{2+}-dependent cellular physiological activity.

The effects of Mn^{2+} on phosphorylation patterns of synaptic plasma membrane proteins are of interest as well. Synaptic activity is regulated by phosphorylation–dephosphorylation cycles [26]. The excessive phosphorylation obtained in the presence of Mn^{2+}, which seems to be independent of the presence of both Ca^{2+} and Mg^{2+}, could affect synaptic communication and also a very large number of other regulatory pathways.

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