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Effects of Mn^{2+} on the responses induced by different spasmogens in the oestrogen-primed rat uterus

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Abstract

We investigated the effect of Mn^{2+} on the mechanical responses evoked by high K^+ (60 mM) or low Na^+ (25 mM) solutions, oxytocin and neurokinin A in the oestrogen-primed rat uterus. In a Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution, high K^+ or low Na^+ solutions produced contractions of smaller amplitude than those observed in a normal Ca^{2+} (0.54 mM) solution, which were abolished by nifedipine (1 μM). Oxytocin (1 μM) and neurokinin A (1 μM, in the presence of phosphoramidon 1 μM) evoked nifedipine-insensitive contractile responses similar to (oxytocin) or smaller (neurokinin A) in amplitude than those observed in Ca^{2+} (0.54 mM)-containing solution. In strips loaded with Ca^{2+} (2.16 mM) for 10 min and then exposed to a Ca^{2+}- and Mn^{2+}-free, EGTA (3 mM)-containing medium for 4 min, both oxytocin and neurokinin A induced transient contraction followed by a small sustained response. The transient component of the response was abolished by cyclopiazonic acid (10 μM). When preparations were loaded with Mn^{2+} (2.16 mM) for 10 min, only the small, tonic contraction was observed. In Ca^{2+}-containing solution, Mn^{2+} (0.01–10 mM) inhibited in a concentration-dependent manner the rhythmic contractions developed either spontaneously or by electrical stimulation as well as high K^+- and neurokinin A-induced contractions. Mn^{2+} also abolished the rhythmic, but not the tonic component of the response to oxytocin, and the preparation remained maximally contracted. These data suggest that in the oestrogen-primed rat uterus, Mn^{2+} acts as an antagonist of Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels. In addition, Mn^{2+} enters the cell mainly through nifedipine-insensitive receptor-operated channels and, to a lesser degree, through L-type Ca^{2+} channels to produce contraction by directly activating the contractile machinery.

Keywords: Mn^{2+}; Ca^{2+} channel, receptor-operated; Uterus, rat

1. Introduction

In the myometrium, an increase in the concentration of free myoplasmic Ca^{2+} is essential for the initiation of contraction (Anwer and Sanborn, 1989; Arnaudeau et al., 1994). The rise in free Ca^{2+} depends both on Ca^{2+} entry from the extracellular fluid and on Ca^{2+} mobilization from intracellular stores, although the relative contribution of these sources of Ca^{2+} to contraction remains unclear and depends, among other factors, on the type of stimulus (Kasai et al., 1994; Ausina et al., 1996a). As far as external Ca^{2+} is concerned, sarcolemmal voltage-gated and agonist-operated Ca^{2+} channels constitute the primary mode of Ca^{2+} entry, although Ca^{2+} influx via Na^+/Ca^{2+} exchange (Savineau et al., 1987; Ausina et al., 1996a) and via the Ca^{2+} leakage pathway (Lalanne et al., 1984) has been reported to occur.

Voltage-operated Ca^{2+} channels can be activated by depolarization of the cell membrane without requiring receptor occupancy. Electrophysiological and molecular cloning studies have revealed multiple types of voltage-operated Ca^{2+} channels, which have a different distribution, voltage dependence, kinetics of activation/inactivation and conductance (Olivera et al., 1994; Catterall, 1995). Among

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them, L-type channels are the best characterized and perhaps the principal Ca^{2+} channels in postsynaptic, peripheral structures, and they play an important role in regulating uterine smooth muscle contraction (Granger et al., 1986). They have been the object of extensive research due, in part, to the availability of highly specific blockers. With respect to receptor-operated Ca^{2+} channels, their presence and importance have been demonstrated in different cell types (Bolton, 1979; Krause et al., 1993). However, their function and primary structure are poorly understood and, so far, no high-affinity blockers exist.

Inorganic cations such as Ni^{2+}, Co^{2+}, Mn^{2+} and La^{3+} have been widely used as blockers of Ca^{2+} influx through L-type Ca^{2+} channels (Tsien et al., 1987). Some of them, i.e., Mn^{2+} and Ni^{2+}, also block Ca^{2+} channels of the T- and R-type (Hofmann et al., 1987; Hong and Chang, 1995). Conversely, very high concentrations of these inorganic cations are necessary to block receptor-mediated Ca^{2+} influx. Moreover, in some, but not all, cell types, some divalent cations can pass through the receptor-mediated Ca^{2+} influx pathway (Krause et al., 1993), thus providing a tool for differentiating voltage- and agonist-sensitive Ca^{2+} channels.

Several reports have been published on the effects of Mn^{2+} on myometrial contractility. In saponin-skinned fibers isolated from pregnant rat myometrium, Mn^{2+} can substitute for Ca^{2+} and acts as an activator of the contractile machinery (Savineau et al., 1988). In intact rat uterus, this cation inhibits the spasmogenic response to acetylcholine, while contractions induced by vanadate and prostaglandin E_2 are only slightly reduced (Mironneau et al., 1984; Sakai et al., 1983). Sakai et al. (1983) observed that oxytocin causes the same extent of contraction of rat myometrium in Ca^{2+}-free solution containing Mn^{2+} as in normal, Ca^{2+}-containing solution. Mn^{2+} is also able to contract the isolated rat myometrium in K^+-rich, Ca^{2+}-free solution (Sakai and Uchida, 1981). Responses to both oxytocin and K^+-rich solution were inhibited by methoxyverapamil, thus suggesting that they were mediated by Mn^{2+} influx via L-type Ca^{2+} channels (Sakai and Uchida, 1981; Sakai et al., 1983). Conversely, Anderson et al. (1971) reported that extracellular Mn^{2+} inhibits Ca^{2+} influx in uterine cells.

The aim of the present study was to investigate further the nature of the actions of Mn^{2+} on mechanical activity in the oestrogen-primed rat uterus. For this purpose, we examined the effect of Mn^{2+} and of Ca^{2+} removal and addition of Mn^{2+} on spontaneous and electrically evoked uterine rhythmic contractions as well as on spasmogenic responses evoked by a high K^+ (60 mM) solution, a low Na^+ (25 mM) solution and oxytocin. We also studied the effect of Mn^{2+} on the response induced by neurokinin A. This tachykinin potently contracts uterine smooth muscle (Pennefather et al., 1993; Fisher et al., 1993) but, to our knowledge, no systematic studies exist on the precise mechanisms involved in this contraction.

2. Materials and methods

2.1. Animals

Myometrial tissue was obtained from virgin female Wistar rats (200–250 g) pretreated with 17β-oestradiol benzoate (20 μg/kg, i.p.) 24 h before the experiment. Rats were killed and myometrial tissue was removed. The oestrus stage was confirmed by microscopic examination of a vaginal smear, taken posthumously.

2.2. Tissue bath experiments

2.2.1. Experimental conditions

Longitudinal strips of uterine smooth muscle (8–10 mm in length and 1–2 mm in width) were prepared and mounted under 0.5 g resting force in silanized (with 5% dimethyl-dichlorosilane in chloroform) tissue baths containing 4 ml of Sund's physiological salt solution (for composition see below). The solution was bubbled continuously with 95% O_2/5% CO_2 and was warmed to 32°C. Mechanical responses were recorded isometrically by means of force-displacement transducers (Grass FT-03) connected to a LETICA amplifier and a ABB GOERZ SE 130 multichannel recorder.

Physiological solutions had the following composition: (a) Sund's physiological salt solution contained (mM): NaCl 154; KCl 5.6; $CaCl_2$ 0.54; $MgCl_2$ 0.95; $NaHCO_3$ 5.95 and glucose 2.78. In some experiments, the concentration of $CaCl_2$ was increased to 1.08 or 2.16 mM. (b) High K^+ (60 mM) solution was prepared by substituting an appropriate concentration of NaCl with equimolar KCl. (c) Low Na^+ (25 mM) solution was made by replacing NaCl with isoosmolar sucrose. (d) Ca^{2+}-free solution was prepared by omitting $CaCl_2$ and adding EGTA at appropriate concentrations. (e) Ca^{2+}-free, Mn^{2+}-containing solution was prepared by replacing $CaCl_2$ with an equimolar concentration of $MnCl_2$. (f) Ca^{2+}-free, K^+-rich, Mn^{2+}-containing solution and Ca^{2+}-free, low Na^+ (25 mM), Mn^{2+}-containing solution were prepared as in (b) and (c), respectively but adding $MnCl_2$ in place of $CaCl_2$.

2.2.2. Protocols

After a 45-min equilibration period, contraction was induced two or more times by administration of a maximally effective concentration of acetylcholine (1 mM) until constant responses were obtained. The last response served as an internal standard for all experiments. Uterine strips were allowed to equilibrate for a further 60 min period in physiological solution (time-matched paired control strips) or in Ca^{2+}-free, Mn^{2+}-containing solution (test tissues). A first set of experiments was performed to assess the ability of uterine strips to develop spontaneous or electrically generated rhythmic contractions in Mn^{2+}-containing solution. Twitch contractions were elicited as described by Pennefather et al. (1990) by field electrical

stimulation delivered through platinum electrodes incorporated into the tissue holders. Trains of pulses (2 ms, 25 V, 30 Hz) were applied for 5 s every 100 s, using a Grass S 11 stimulator connected to a multiplier (Stimu-splitter II, Med-Lab). Electrical stimulation was carried out in the continuous presence of tetrodotoxin (1 μM) to avoid a possible contribution of neurogenic mechanisms to the observed responses.

In a second set of experiments contraction was obtained by exposure to high K^+ (60 mM) or low Na^+ (25 mM) solutions or to maximally effective concentrations of oxytocin (1 μM) or neurokinin A (1 μM) in preparations bathed in Ca^{2+} (0.54 mM)- or in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution. Experiments with neurokinin A were carried out in the presence of the neutral endopeptidase inhibitor phosphoramidon (*N*-(α-L-rhamnopyranosyl-oxyhydroxyphosphinyl)-L-leucyl-L-tryptophan, 1 μM), added to the bath 20 min before the agonist. Phosphoramidon had no effect on the oxytocin (1 μM)-induced contraction. Some experiments were carried out in the presence of the L-type Ca^{2+} channel blocker nifedipine (1 μM), which was added to the bath 20 min before spasmogens and remained in contact with the preparation during the generation of the response. In other experiments, three successive responses to spasmogens were obtained at 60–100-min intervals in strips maintained in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution throughout the experiment, either in the absence or in the presence of nifedipine (1 μM).

In a third set of experiments, we investigated the possible involvement of intracellular Ca^{2+} in uterine contractions and whether Mn^{2+} might act as a substitute for Ca^{2+} at intracellular storage sites. The uterine strips were incubated in physiological solution containing 2.16 mM Ca^{2+} or in Ca^{2+}-free, 2.16 mM Mn^{2+}-containing solution for 10 min (Ca^{2+} or Mn^{2+} loading period), after which they were exposed to a Ca^{2+}- and Mn^{2+}-free solution containing 3 mM EGTA for 4 min before application of spasmogens. After washout of the contractile agent, tissues were maintained for 15 min in Ca^{2+}- and Mn^{2+}-free solution containing 0.3 mM EGTA. We found in preliminary experiments that the same response could be obtained repeatedly from one strip when agonists were applied every 34 min after the same Ca^{2+} loading procedure (Ausina et al., 1996b). In experiments performed to determine the influence of exposure to an agonist on the subsequent response to another agonist, the first agonist was left in contact with the preparation for 20 s and the second agonist was added 1 min after washout of the first agent. Some experiments were performed in the presence of cyclopiazonic acid, an inhibitor of the Ca^{2+}-ATPase of the sarcoplasmic reticulum which has been shown to inhibit Ca^{2+} uptake into the intracellular stores in rat myometrium (Kasai et al., 1994). Cyclopiazonic acid (10 μM) was added after the loading time or applied 2 min before and remained in contact with the preparation during the loading period. The effect of cyclopiazonic acid on the successive contractions induced by oxytocin or neurokinin A in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution in the presence of nifedipine was also assessed.

A fourth set of experiments was carried out to examine the effects of Mn^{2+} on uterine contractions in Ca^{2+}-containing solution. When the tonic contraction induced by KCl was sustained or when the rhythmic contractions evoked spontaneously, electrically or by exposure to oxytocin or neurokinin A had attained a constant amplitude, saline (time-matched control tissues) or Mn^{2+} (0.01–10 mM, test tissues) was added to the bath in a cumulative manner at 10-min intervals. A single concentration-response curve for Mn^{2+} was made for each tissue segment.

2.3. Inositol phosphate determination

The determination of total inositol phosphate accumulation was adapted from Berridge et al. (1982) and Irvine et al. (1985). Oestrogen-primed uteri were excised from 8 rats and exposed to Sund's solution containing 2 μCi of *myo*-[2-^{3}H]inositol/ml buffer for 4 h at 37°C and gassed with O_2 plus CO_2 mixture. After this incubation, the tissue was washed four times with 25 ml of physiological solution. Five tubes were prepared: two tubes containing 1 ml of Sund's solution, two tubes with 1 ml of Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution and one tube containing 1 ml of Ca^{2+}- and Mn^{2+}-free solution. Each individual tube contained 0.3–0.6 g of tissue from eight different rats and was incubated at 32°C for 1 h. LiCl (10 mM) was added 30 s before the agonist to inhibit metabolism of inositol monophosphates (Berridge et al., 1982).

Stimulation by the corresponding salt solution (control tubes) or oxytocin (1 μM) in either Ca^{2+} (0.54 mM)-containing or Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution was performed for 30 min. Basal levels of inositol phosphates were also measured in Ca^{2+}-free solution. Stimulations were stopped by placing the tubes in a cold water bath (4°C) and adding 1.5 ml of a refrigerated mixture of chloroform/methanol/10 M HCl (100:200:4, v/v/v) with vigorous shaking. The samples were centrifuged ($4000 \times g$) for 10 min at 4°C and the aqueous phases were stored at −20°C until analysis. Separation of total [^{3}H]inositol phosphates was performed with an HPLC ion-exchange system, using a 0.46×25 cm Partisil SAX 10 high pressure anion exchange column (Life Sciences International, Cergy-Pontoise, France), at a flow rate of 1.3 ml/min. The samples were brought to pH 4 with 50 μl of 1.2 M ammonium formate and then loaded (500 μl) onto the column. Distilled water was used as eluant for 6 min to elute inositol, and then a linear gradient from 0 to 100% of potassium phosphate 1.0 M buffered to pH 3.7 with orthophosphoric acid over 24 min was used. Radioactivity was detected by a Flow-One on-line radioactivity flow detector (Packard, Meriden, CT, USA) equipped with a

2-ml liquid flow cell (sensitivity 1500 dpm/mμCi). Results (dpm/g tissue wet weight) are expressed in terms of total phosphoinositides.

2.4. Drugs

Oxytocin, acetylcholine hydrochloride, phosphoramidon (*N*-(α-L-rhamnopyranosyl-oxyhydroxyphosphinyl)-L-leucyl-L-tryptophan sodium salt), nifedipine, cyclopiazonic acid and tetrodotoxin were from Sigma (St. Louis, MO, USA). Neurokinin A was from Bachem (Switzerland). All the other reagents were of analytical grade. Cyclopiazonic acid and nifedipine were dissolved in dimethyl sulphoxide and absolute ethanol, respectively, and then diluted with deionized water to appropriate concentrations. The final dimethyl sulphoxide or ethanol concentration in the bathing medium was lower than 0.1% and had no effect on mechanical responses. The other agents were dissolved in deionized water.

2.5. Expression and statistical analysis of results

Contractile responses were measured in different ways due to their complex nature. Phasic contractions represent the contractile tension at the peak of the first component, before tension reaches a plateau (KCl 60 mM and Na^+ 25 mM) or prior to the development of rhythmic oscillations (oxytocin and neurokinin A). Tonic contractions refer to the steady-state tension at the plateau (KCl 60 mM and Na^+ 25 mM) or at the bottom of the descending phase of rhythmic contractions (oxytocin and neurokinin A, calculated as the mean of a 10-min period). Phasic and tonic responses are expressed as percentages of the contractile tension at the peak of the first component of the acetylcholine-induced contraction. To assess the effects of Mn^{2+} on the contractile responses induced by spasmogens in Ca^{2+}-containing solution, the maximal amplitude of spontaneous or electrically induced rhythmic contractions or the area under the force-time curve for high K^+, oxytocin and neurokinin A was measured for 10 min before and during the period that each concentration of Mn^{2+} was in contact with the preparation. Areas were measured from polygraph tracings using a digitizing tablet and the Sigma-Scan software package (Jandel Scientific). In each segment of tissue, the response (amplitude or area) measured for 10 min after addition of each concentration of Mn^{2+} is expressed as a percentage of the response (amplitude or area) measured in the 10-min period prior to the administration of Mn^{2+}, to determine the percent contraction remaining. To account for the decline in contractions during measurement of the concentration-response curve for Mn^{2+}, the mean percent contraction remaining was calculated at each time point in paired, time-matched controls. For each Mn^{2+}-exposed tissue the percent contraction remaining at each Mn^{2+} concentration is expressed as a percentage of the mean percent contraction remaining at the corresponding time point in the time-matched controls, as described by Hughes and Hollingsworth (1995). All values in the text and tables are expressed as means ± S.E.M. for *n* number of experiments with uteri from *n* different animals. Statistical significance of differences between two means was assessed by Student's *t*-test for paired or unpaired data. Multiple means were compared by one-way analysis of variance (ANOVA) followed, when ANOVA revealed significant differences, by Bonferroni's test. *P* values of less than 0.05 were considered to represent significant differences.

3. Results

3.1. Contractile responses in Ca^{2+}- and Mn^{2+}-containing solutions

In Ca^{2+}-containing solution, the uterus of oestrogen-treated rats exhibited mechanical oscillatory activity in the absence of exogenous stimuli. Phasic, reproducible and Ca^{2+} concentration-dependent contractions could also be induced by electrical field stimulation in the presence of tetrodotoxin (1 μM), which indicates their myogenic origin. Nifedipine (1 μM) suppressed spontaneous and electrically induced rhythmic contractions (not shown). Un-

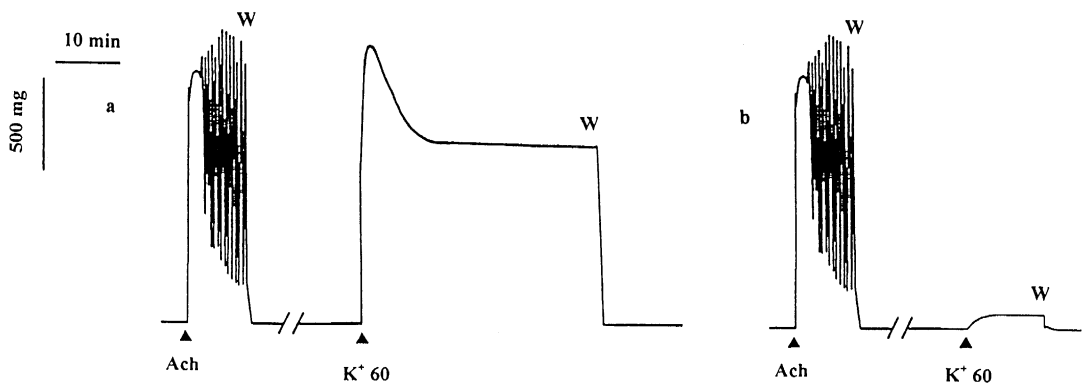

Fig. 1. Typical tracings showing the effect of an isoosmotic high K^+ (60 mM) solution (K^+) on the mechanical activity of oestrogen-primed rat uterus. Responses were obtained in Ca^{2+} (0.54 mM)-containing solution (a) or in a Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution (b). ACh: reference contraction produced by acetylcholine 1 mM in a Ca^{2+} (0.54 mM)-containing solution. Traces are representative of results of four experiments.

Table 1
Maximal contractile responses induced by high K^+ solution (KCl 60 mM), low Na^+ solution (NaCl 25 mM), oxytocin (OXY) and neurokinin A (NKA) of the rat uterus in Ca^{2+} (0.54 mM)-containing solution or in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution, either in the absence or in the presence of nifedipine (NIF)

Pretreatment	Agent	Ca^{2+} (0.54 mM) E_{max} (%)			Ca^{2+}-free, Mn^{2+} (0.54 mM) E_{max} (%)		
		n	Phasic response	Tonic response	*n*	Phasic response	Tonic response
Saline	KCl 60 mM	22	103.0 ± 4.1	92.1 ± 4.7	4	0	12.9 ± 1.3 [a]
	NaCl 25 mM	23	107.3 ± 2.7	3.4 ± 0.5	8	17.1 ± 2.8 [a]	12.2 ± 3.2 [a]
	OXY 1 μM	25	101.3 ± 2.8	37.8 ± 6.3	8	109.6 ± 6.5	119.3 ± 7.4 [a]
	NKA 1 μM	13	94.3 ± 4.2	13.8 ± 4.3	10	37.4 ± 5.8 [a]	34.3 ± 5.7 [a]
NIF 1 μM	KCl 60 mM	4	0	0	4	0	0
	NaCl 25 mM	5	6.0 ± 2.3 [a]	1.3 ± 0.2	5	0	0
	OXY 1 μM	8	80.3 ± 5.9 [a]	42.9 ± 6.2	8	101.1 ± 3.6	117.2 ± 4.9 [a]
	NKA 1 μM	6	33.9 ± 8.3 [a]	2.5 ± 1.9 [a]	6	28.8 ± 5.7 [a]	24.5 ± 4.2 [a]

Values (mean ± S.E.M.) are expressed as a percentage of the contractile tension at the peak of the initial, phasic response to acetylcholine (1 mM) in Ca^{2+} (0.54 mM)-containing solution. Phasic response = mean amplitude at the peak of the initial, phasic component. Tonic response = mean amplitude of the tonic component, measured 30 min after addition of the spasmogen. Significant differences from control responses in Ca^{2+} (0.54 mM)-containing solution: [a] $P < 0.01$, one-way ANOVA. *n* = number of experiments in *n* different animals.

stimulated or electrically stimulated uterine strips were quiescent in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution, and contractility was not restored by raising the external Mn^{2+} concentration to 2.16 mM (not shown).

3.1.1. Response to high K^+ (60 mM) solution

In Ca^{2+} (0.54 mM)-containing solution, isoosmotic addition of KCl (60 mM) caused a rapid, phasic contraction which then decreased and was followed by a prolonged sustained plateau (Fig. 1a, Table 1). This response disappeared in Ca^{2+}-free, EGTA (3 mM)-containing solution. In Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution, a monophasic, slowly developing response of significantly smaller amplitude was observed (Fig. 1b, Table 1). Several successive responses to high K^+ (60 mM) could be obtained in strips bathed in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution without there being a significant change in amplitude (not shown). Nifedipine (1 μM) abolished the response evoked by 60 mM K^+ in both Ca^{2+} and Mn^{2+}-containing solutions (Table 1).

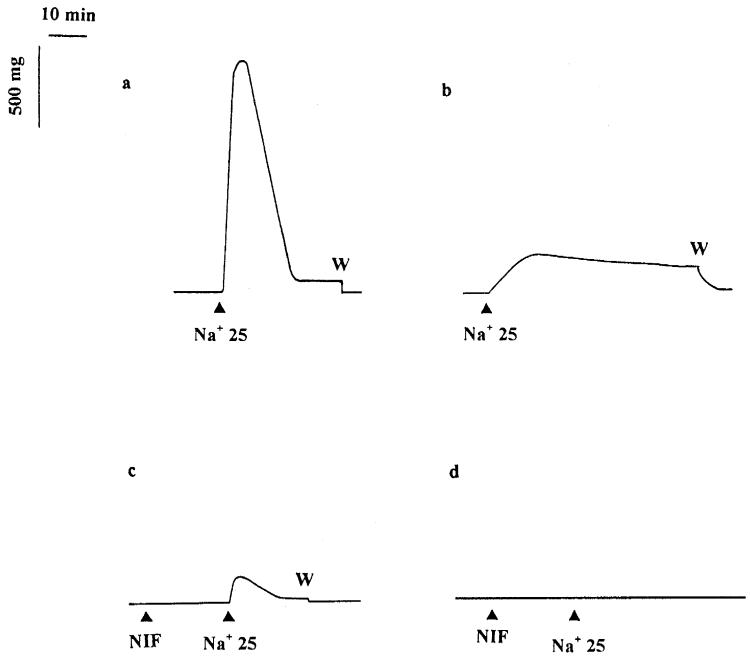

Fig. 2. Typical tracings showing the effect of a low Na^+ (25 mM) solution (Na^+ 25) on the mechanical activity of oestrogen-primed rat uterus. Responses were obtained in Ca^{2+} (0.54 mM)-containing solution (left panels) or in a Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution (right panels) either in the absence (a,b) or in the presence (c,d) of nifedipine (NIF, 1 μM), which was added to the bath 20 min before the low Na^+ solution. Traces are representative of results of 5–23 experiments.

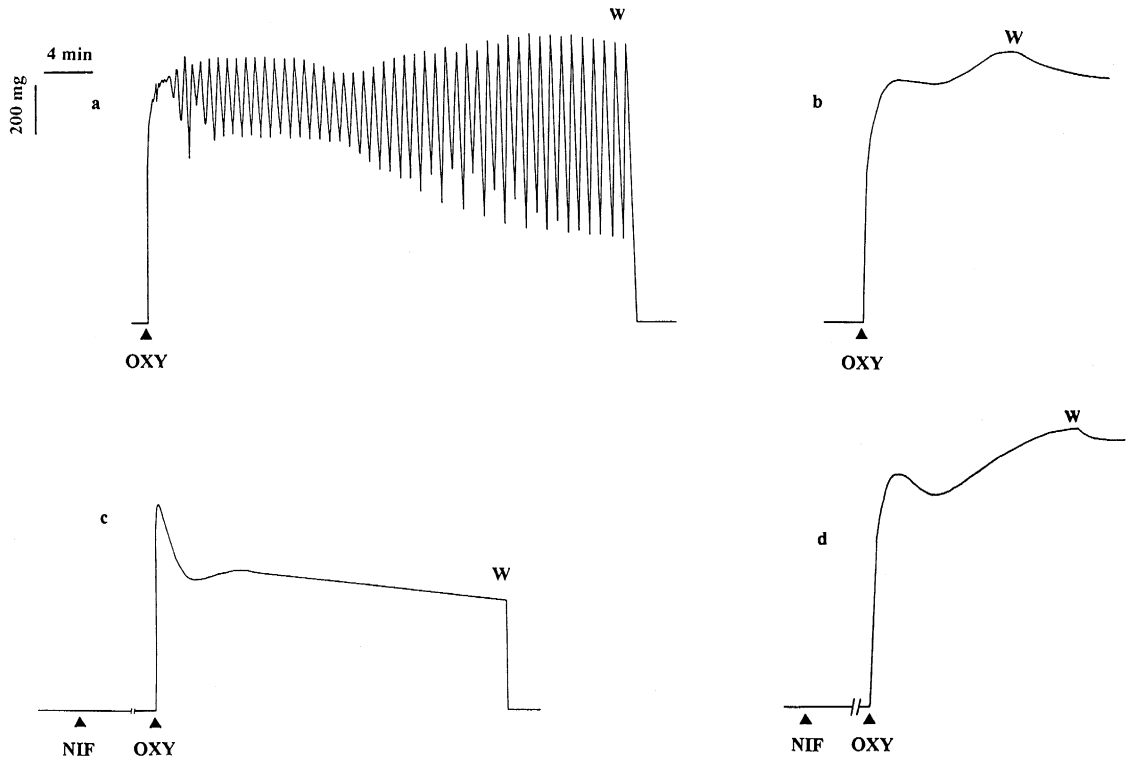

Fig. 3. Typical tracings showing the effect of oxytocin (OXY, 1 μM) on the mechanical activity of oestrogen-primed rat uterus. Responses were obtained in Ca^{2+} (0.54 mM)-containing solution (left panels) or in a Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution (right panels) either in the absence (a,b) or in the presence (c,d) of nifedipine (NIF, 1 μM), which was added to the bath 20 min before OXY. Traces are representative of results of 8–25 experiments.

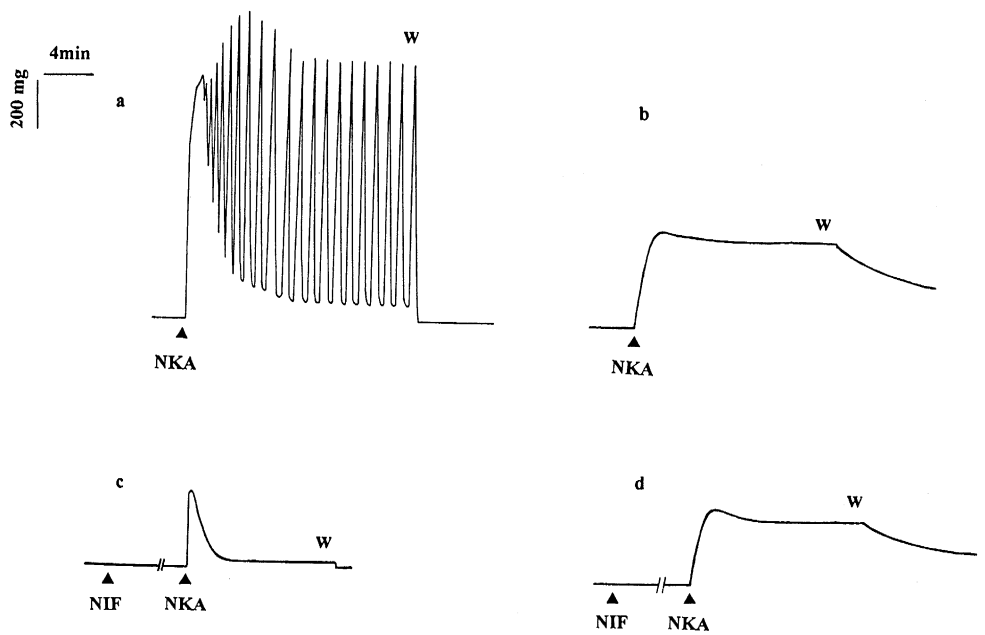

Fig. 4. Typical tracings showing the effect of neurokinin A (NKA, 1 μM) on the mechanical activity of oestrogen-primed rat uterus. Responses were obtained in Ca^{2+} (0.54 mM)-containing solution (left panels) or in a Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution (right panels) in the absence (a,b) or in the presence (c,d) of nifedipine (NIF, 1 μM), which was added to the bath 20 min before NKA. The neutral endopeptidase inhibitor phosphoramidon (1 μM) was added to the bath 20 min before NKA. Traces are representative of results of 6–13 experiments.

3.1.2. Response to low Na^+ (25 mM) solution

In Ca^{2+}-containing solution, reduction of $[Na^+]_o/[Na^+]_i$ gradient by lowering $[Na^+]_o$ to 25 mM caused a strong, transient contraction followed by a small contraction which was sustained during exposure to the modified solution and quickly disappeared after washout (Fig. 2a, Table 1). This contraction was abolished in Ca^{2+}-free, EGTA (3 mM)-containing solution. In Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution, Na^+ (25 mM) caused a contraction smaller than that observed in Ca^{2+}-

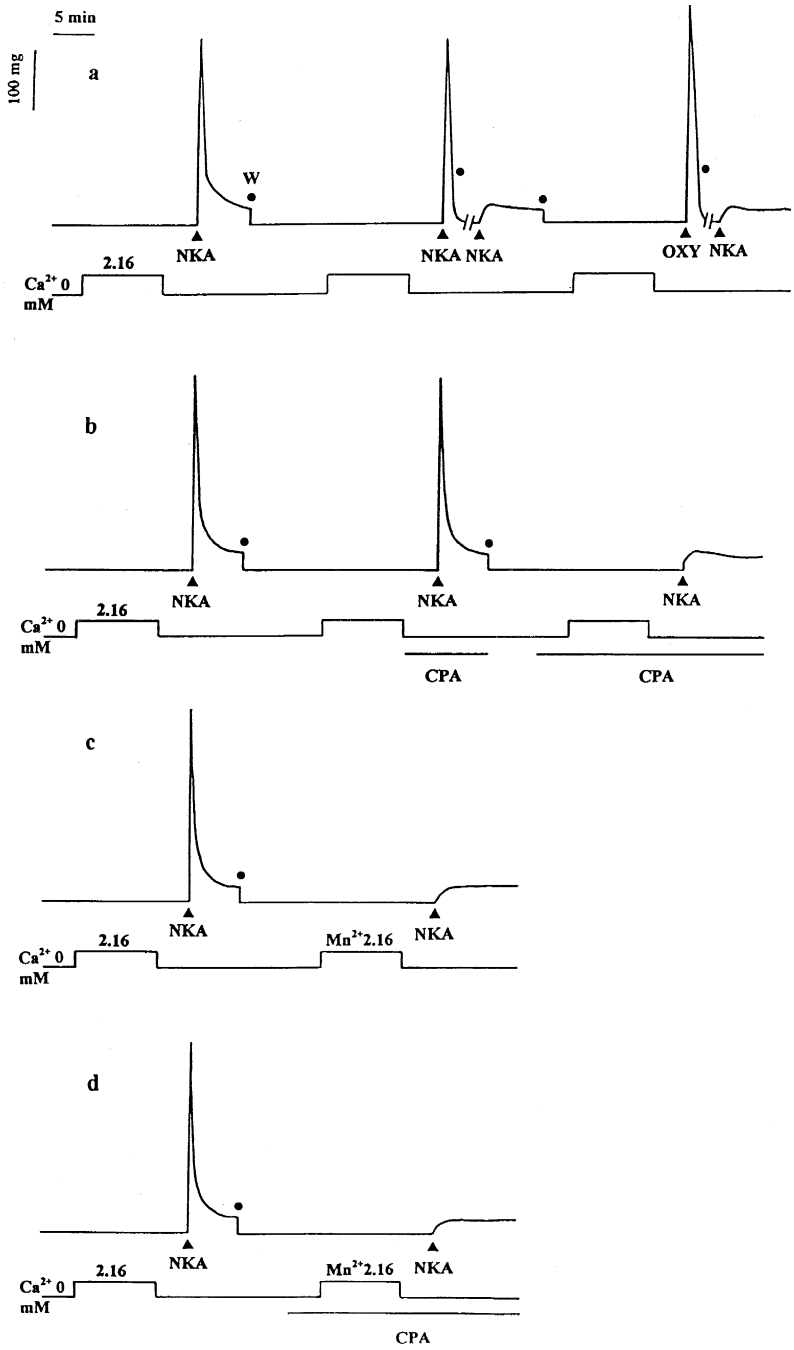

Fig. 5. Representative tracings showing the contractile responses induced by neurokinin A (NKA, 1 μM) in rat uterine strips after short exposure to a Ca^{2+}-free medium. In a and b, Ca^{2+} loading was performed in physiological solution containing 2.16 mM Ca^{2+} for 10 min. NKA was applied after 4-min exposure of strips to a Ca^{2+}-free solution containing EGTA 3 mM and induced a transient contraction followed by a well sustained, small contraction. (a) When NKA was applied in Ca^{2+}-free solution 1 min after exposure to NKA (1 μM) or to oxytocin (1 μM) for 20 s, only the sustained component was observed. The transient response reappeared after a Ca^{2+} loading period. (b) Cyclopiazonic acid (CPA, 10 μM) did not modify the response to NKA when added after Ca^{2+} loading but abolished it when added 2 min before and left in contact with the preparation during the loading period. (c,d) The transient response did not reappear after loading the preparation with Ca^{2+}-free, Mn^{2+} (2.16 mM)-containing solution, either in the absence (c) or in the presence (d) of CPA. Traces are representative of results of 4–6 experiments.

containing solution, with a peak reached at a slower rate of contraction followed by a greater residual contraction (Fig. 2b, Table 1). This contraction slowly disappeared after washout; it took 3–5 min to recover the initial resting tension (Fig. 2b). This response could be reproduced several times in the same uterine strip, with an interval of 60 min between responses (not shown). As illustrated in Fig. 2 (c and d), a small portion of the contraction elicited by Na^+ (25 mM) in Ca^{2+}-containing solution persisted in the presence of nifedipine (1 μM), while no response was observed in Mn^{2+}-containing solution.

3.1.3. Response to oxytocin and neurokinin A

The responses induced by maximally effective concentrations of oxytocin (1 μM) and neurokinin A (1 μM) in Ca^{2+}-containing solution are shown in Figs. 3 and 4. The two agonists elicited an initial rapid phasic contraction of similar amplitude (Table 1) which reached a peak within 58 ± 5 and 78 ± 4 s, respectively, and was then followed by a tonic response with superimposed rhythmic contractions (Fig. 3a and Fig. 4a). Nifedipine (1 μM) abolished the rhythmic oscillations evoked by both agonists but was significantly more effective as an inhibitor of the neurokinin A-induced phasic response than of the oxytocin-induced one (Fig. 3c and Fig. 4c, Table 1). Moreover, in the presence of nifedipine, the residual phasic response to neurokinin A was followed by a relatively rapid return to a tone slightly higher than the resting tone, while in the case of oxytocin, the initial, phasic contraction declined partially and was followed by a tonic contraction which slowly decreased with time. In Ca^{2+}-free, Mn^{2+}-containing solution the rhythmic contractions accompanying responses to oxytocin and neurokinin A disappeared, and both agonists induced sustained contractions which were not significantly affected by nifedipine (1 μM, Fig. 3b,d and Fig. 4b,d, Table 1). The oxytocin-induced phasic contraction was similar in amplitude to that observed in a Ca^{2+}-containing solution, while the response to neurokinin A was smaller (Fig. 3b and Fig. 4b, Table 1). The time needed to reach the peak of contraction was significantly increased (9.0 ± 0.5 min and 5.0 ± 0.5 min for oxytocin and neurokinin A, respectively). These responses were difficult to remove after washout of the agonist, and the resting tension returned to previous levels after 80–90 min in the case of oxytocin and after 30–40 min in the case of neurokinin A. Nevertheless, agonist-induced contractions were highly reproducible and three or more successive responses could be obtained at 60 (neurokinin A)- or 100 (oxytocin)-min intervals in the same uterine strip, maintained in Ca^{2+}-free, Mn^{2+}-containing solution either in the absence or in the presence of nifedipine (not shown). Similar contractile responses were observed when a higher concentration of oxytocin (3 μM) or neurokinin A (10 μM) was tested (not shown).

After 4-min exposure to a Ca^{2+}-free solution containing EGTA 3 mM, oxytocin (1 μM) and neurokinin A (1 μM) induced a transient contraction ($14.5 \pm 1.1\%$, $n = 16$ and $11.3 \pm 1.2\%$, $n = 6$, of the initial phasic response to acetylcholine in 0.54 mM Ca^{2+}-containing solution, respectively) followed by a small sustained response ($5.1 \pm 0.4\%$, $n = 16$ and $2.2 \pm 0.3\%$, $n = 6$ of the initial response to acetylcholine for oxytocin and neurokinin A, respectively). When oxytocin (not shown) or neurokinin A (Fig. 5a) was applied a second time in Ca^{2+}-free medium 1 min after exposure to the same or to the other agonist for 20 s, only the sustained component was observed. However, the transient response reappeared and could be reproduced several times in the same uterine strip by repeating the Ca^{2+} loading procedure (Fig. 5, not shown for oxytocin). This demonstrated the dependence of the transient response on Ca^{2+} release from uterine internal stores. The involvement of intracellular Ca^{2+} in these responses was further assessed by studying the effects of cyclopiazonic acid (10 μM). This inhibitor of the Ca^{2+}-ATPase of the sarcoplasmic reticulum did not modify the transient response to oxytocin (not shown) or neurokinin A (Fig. 5b)

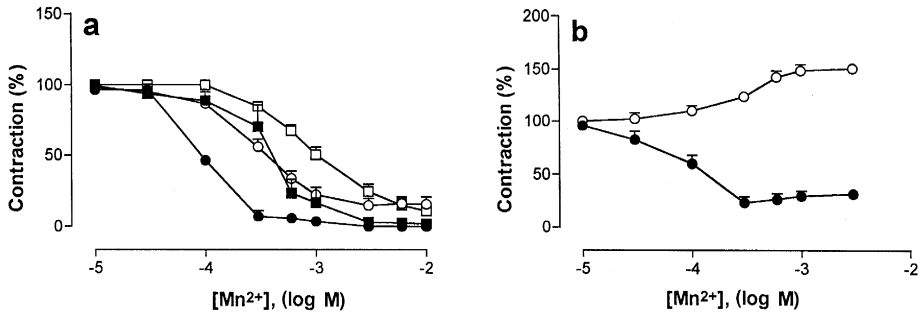

Fig. 6. Concentration–response curves for Mn^{2+} on the spontaneous contractions or on the contractions induced by electrical field stimulation or by various spasmogens of the rat uterus in Ca^{2+}-containing solution. (a) Effect of Mn^{2+} on electrically induced contractions in Ca^{2+} (0.54 mM)-containing solution (●), on spontaneous rhythmic contractions developed in Ca^{2+} (2.16 mM)-containing solution (■) and on the contraction induced by a high K^+ (60 mM) solution in Ca^{2+} (0.54 mM (○) or 2.16 mM (□))-containing medium. (b) Effect of Mn^{2+} on the contractions induced either by neurokinin A (1 μM (●)) or by oxytocin (1 μM (○)) in Ca^{2+} (0.54 mM)-containing medium. Data (mean ± S.E.M. of four or six experiments) represent increases in tension (spontaneous and electrically induced rhythmic contractions), expressed as a percentage of the maximal tension developed in the absence of Mn^{2+} or areas under the force-time curve (high K^+, oxytocin and neurokinin A), expressed as a percentage of that measured during a 10-min period before Mn^{2+} addition and then corrected relative to the percent contraction remaining in time-matched paired control strips.

when applied after Ca^{2+} loading but abolished it when added 2 min before the loading period and left in contact with the preparation until agonist application.

The transient response did not reappear when the loading solution contained 2.16 mM Mn^{2+} instead of Ca^{2+} (Fig. 5c, not shown for oxytocin). Under these conditions, the agonists induced only the small tonic contraction, regardless of whether Mn^{2+} loading was carried out in the absence or in the presence of cyclopiazonic acid (Fig. 5c,d). We also studied the effect of cyclopiazonic acid on the contraction induced by oxytocin or neurokinin A in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution. Three successive responses to both agonists were not significantly different when obtained in the absence or in the presence of cyclopiazonic acid (10 μM, not shown).

3.2. Effect of Mn^{2+} on established uterine contractions in Ca^{2+}-containing solution

In these experiments, we studied the effect elicited by Mn^{2+} in uterine strips maintained in Ca^{2+}-containing solution and contracted by different agents. The area under the force-time curve for the contractions induced by high K^+ (60 mM) solution in medium containing 0.54 or 2.16 mM Ca^{2+}, by oxytocin and by neurokinin A (measured from the time needed to reach a stable response: 30–45 min for high K^+ in 0.54 mM Ca^{2+} solution, 15–30 min for high K^+ in 2.16 mM Ca^{2+} solution, oxytocin and neurokinin A) declined by $22.9 \pm 1.6\%$ ($n = 6$), $10.7 \pm 2.3\%$ ($n = 4$), $27.2 \pm 6.5\%$ ($n = 6$) and $20.1 \pm 7.9\%$ ($n = 6$), respectively, in the time needed to measure the dose-response curve for Mn^{2+} (1 h approximately) in time-matched paired control strips. Rhythmic contractions evoked electrically were sustained during the period of observation (up to 2 h). As spontaneous rhythmic contractions usually change in amplitude or disappear with time, only two cumulative concentrations of Mn^{2+} were assayed on each strip. Addition of cumulative amounts of $MnCl_2$ (0.01–10 mM) on the sustained contraction induced by KCl evoked concentration-dependent relaxation (Fig. 6a). $MnCl_2$ also inhibited in a concentration-dependent manner the uterine rhythmic contractions elicited spontaneously or electrically (Fig. 6a) or by exposure to neurokinin A (Fig. 6b). As shown in Fig. 6, the potency of Mn^{2+} as a relaxant depended both on the type of stimulus and on the concentration of external Ca^{2+}. Fig. 6 also shows that a fraction of the response to KCl and neurokinin A was resistant to relaxation. Mn^{2+} progressively decreased and finally abolished the rhythmic contractions evoked by oxytocin and neurokinin A. However, Mn^{2+} in concentrations up to 10 mM failed to suppress the tonic component of the response to oxytocin (Fig. 6b).

3.3. Influence of Mn^{2+} on oxytocin-induced increase in [^{3}H]inositol phosphate formation

The basal level of [^{3}H]inositol phosphates was similar in both Ca^{2+} (0.54 mM)-containing and Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solutions. Conversely, basal levels of inositol phosphates were significantly lower after 1 h incubation in Ca^{2+}-free solution (Fig. 7). Oxytocin (1 μM) produced a similar and significant increase in total [^{3}H]inositol phosphates above basal levels in myometrial segments incubated in either Ca^{2+} (0.54 mM)-containing solution or Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution (Fig. 7).

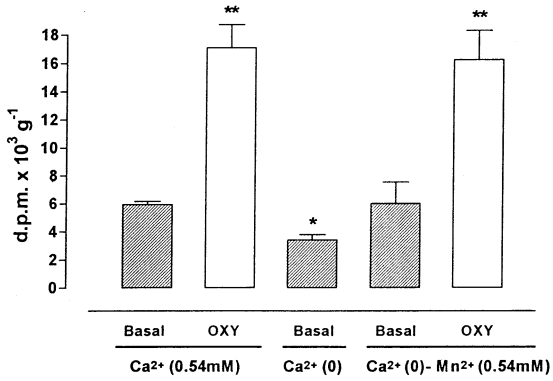

Fig. 7. Total [^{3}H]inositol phosphate production of oestrogen-primed rat uterus in response to oxytocin (1 μM) in Ca^{2+} (0.54 mM)-containing solution or in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution. The results (means ± S.E.M.) are expressed in d.p.m. × 1000 g^{-1} of tissue wet weight. Significant differences in total [^{3}H]inositol phosphate formation from basal values in Ca^{2+} (0.54 mM)-containing solution: * $P < 0.05$; ** $P < 0.01$. Number of experiments was three, each experiment carried out with uteri from eight different animals.

4. Discussion

The present study shows that Mn^{2+} may have both inhibitory and stimulatory properties on the contractions elicited in oestrogen-primed rat myometrium. Traditionally, Mn^{2+} is considered an inorganic Ca^{2+} antagonist (Tsien et al., 1987; Anderson et al., 1971). However, Mn^{2+} is known to enter cells through divalent cation channels in the plasmalemma including the non-regulated leakage pathway (Chen and Van Breemen, 1993), voltage-operated Ca^{2+} channels (Sakai and Uchida, 1981; Sakai et al., 1983; Ushijima and Gomi, 1996) and receptor-operated Ca^{2+} channels (Chen and Van Breemen, 1993; Krause et al., 1993).

Rhythmic contractions of rat myometrium in vitro may be obtained either spontaneously or by electrical stimulation. These contractions were abolished in the presence of L-type Ca^{2+} channel blockers, by removal of external Ca^{2+} or by incubation in Ca^{2+}-free, Mn^{2+} (0.54, 1.08 or 2.16 mM)-containing medium (Lalanne et al., 1984; this study). In addition, Mn^{2+} concentration dependently inhibited the rhythmic contractions obtained in preparations bathed in Ca^{2+} (0.54 or 2.16 mM)-containing solution (this study). These data indicate that Mn^{2+} acts mainly by interfering with Ca^{2+} entry through voltage-operated Ca^{2+} channels, as previously reported in other studies (Anderson et al., 1971).

Addition of an isoosmotic high K^+ (60 mM) solution results in a maximal contraction of rat myometrium (Falk, 1991; Ausina et al., 1996b) which is suppressed in a Ca^{2+}-free medium or in the presence of nifedipine (1 μM). The contraction induced by high K^+ (60 mM) was greatly reduced in preparations bathed in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution, although a residual, nifedipine-sensitive contraction persisted. In contrast with this spasmogenic activity, Mn^{2+} inhibited, in a concentration-related fashion, the contraction produced by K^+ (60 mM) in rat myometrium bathed in Ca^{2+} (0.54 or 2.16 mM)-containing solution. Again, a residual contraction to KCl remained in the presence of maximal concentrations of Mn^{2+}. This indicates that responses observed in Ca^{2+}-free, Mn^{2+}-containing solution are a consequence of addition of Mn^{2+} and not of Ca^{2+} deletion and lends further support to the suggestion that Mn^{2+} mainly interferes with Ca^{2+} entry through voltage-operated Ca^{2+} channels, producing inhibition of KCl-induced contraction. Additionally, Mn^{2+} also seems to be able to enter through the same channels, although to a lesser degree, and produces a small spasmogenic effect.

Exposure to low Na^+ (25 mM) solution resulted in a transient contraction of rat myometrium that was suppressed in Ca^{2+}-free solution and was markedly reduced but not completely abolished in nifedipine (1 μM)-treated tissues. This suggests that Ca^{2+} entry by nifedipine-sensitive Ca^{2+} channels as well as by the nifedipine-insensitive Na^+/Ca^{2+} counterexchange mechanism is involved in the generation of the contractile response to Na^+-deficient medium, as previously reported for rat myometrium (Savineau et al., 1987). As was the case for a high K^+ solution, a small contraction was observed when exposure to low Na^+ solution was carried out in Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution. This contraction was completely inhibited by nifedipine (1 μM), which indicates that the mechanism involved was Mn^{2+} entry through L-type Ca^{2+} channels rather than entry in exchange for Na^+.

The effects of Mn^{2+} were also explored by using two different uterotonic agents, oxytocin and neurokinin A. These two agonists produce a complex response consisting of an initial phasic contraction followed by a tonic contraction with superimposed rhythmic activity. Nifedipine (1 μM) abolished the rhythmic contractions accompanying responses to both agonists in Ca^{2+} (0.54 mM)-containing solution. However, nifedipine only slightly reduced the amplitude of the oxytocin-induced phasic and tonic responses (Hughes and Hollingsworth, 1995; this study) while affecting to a much greater degree the neurokinin A-induced ones. Our results also show that both agonists induced small, phasic responses similar in amplitude after 4-min exposure to a Ca^{2+}-free, EGTA (3 mM)-containing solution. These data confirm that Ca^{2+} influx through nifedipine-insensitive Ca^{2+} channels plays an important role in mediating the contraction to oxytocin (Anwer and Sanborn, 1989). In contrast, the neurokinin A-induced contracture appears more dependent on Ca^{2+} influx through L-type Ca^{2+} channels. In Ca^{2+}-free, Mn^{2+} (0.54 mM)-containing solution, agonist-induced rhythmic contractions were absent, demonstrating again that Mn^{2+} is not an efficient substitute for Ca^{2+} in the production of these contractions. The fact that oxytocin elicited a contraction of similar amplitude but at a slower rate compared to contractions in Ca^{2+}-containing medium confirms previous data from Sakai et al. (1983). In Ca^{2+}-free medium, Mn^{2+} ions supported a contraction in response to neurokinin A that was, however, smaller than the response obtained in the presence of Ca^{2+}. Nifedipine did not significantly affect the responses to either agonist. It thus appears likely that nifedipine-insensitive channels are opened by oxytocin and, to a lesser extent, by neurokinin A, and that Mn^{2+} ions gain access through these cation channels to produce contraction. In some cell types, i.e., human platelets and umbilical vein endothelial cells, intracellular Ca^{2+} release causes the opening of a channel in the sarcolemma that is permeable to Mn^{2+} ions (Jacob, 1990; Alonso et al., 1991). Hence, the activation of the Mn^{2+} influx pathway in uterine cells may be linked to depletion of intracellular Ca^{2+} stores. Alternatively, this pathway may be activated as a consequence of receptor occupancy. The present data show that neurokinin A caused phasic responses of only slightly lower amplitude than oxytocin in Ca^{2+}-free solution and that the response to each agonist was abolished by previous exposure to the other agonist. This suggests that oxytocin and neurokinin A mobilized Ca^{2+} from a common intracellular Ca^{2+} pool in rat myometrium. However, only oxytocin caused a full response in Mn^{2+}-containing solution. It thus appears that the Mn^{2+} entry pathway is activated as a direct consequence of agonist-receptor interaction rather than as a consequence of intracellular Ca^{2+} release. Moreover, when Mn^{2+} was added when there was an established contraction induced by oxytocin or neurokinin A in Ca^{2+} (0.54 mM)-containing solution the preparation remained maximally (oxytocin) or partially (neurokinin A) contracted, suggesting that the Mn^{2+} entry pathway remains open during exposure to the agonist. Finally, we examined whether Mn^{2+} can replace Ca^{2+} at the intracellular storage sites, thus contributing to the contractions induced by these agonists in Ca^{2+}-free, Mn^{2+}-containing solution. A unique inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} store has been described in myometrial cells of rats (Savineau et al., 1988; Kasai et al., 1994). In our study, the basal levels of total [^{3}H]inositol phosphates were similar in Ca^{2+}-containing and Ca^{2+}-free, Mn^{2+}-containing solutions but were significantly lower in uteri exposed to a Ca^{2+}- and Mn^{2+}-free solution. Oxytocin produced a similar, significant increase in the intracellular level of total [^{3}H]inositol phosphates in both Ca^{2+}-containing and Ca^{2+}-free, Mn^{2+}-containing solutions. After Ca^{2+} loading, preparations bathed in Ca^{2+}-free EGTA-containing medium responded with a transient

contraction followed by a small sustained contraction when challenged with oxytocin or neurokinin A. The transient contraction was highly sensitive to the presence of cyclopiazonic acid (a Ca^{2+}-ATPase inhibitor in sarcoplasmic reticulum membrane; Seidler et al., 1989) during Ca^{2+} loading (Kasai et al., 1994; this study). Mn^{2+} was unable to substitute for Ca^{2+} in this process. This and the observation that the successive contractions induced by oxytocin or neurokinin A in Ca^{2+}-free, Mn^{2+}-containing solution were unaffected when elicited in the continuous presence of cyclopiazonic acid suggest that Mn^{2+} ions are not taken up into the sarcoplasmic reticulum by the Ca^{2+} ATPase, as indicated by Gomes da Costa and Madeira (1986).

The response evoked by agonists in Mn^{2+}-containing solution may be due, at least partially, to the increased availability of intracellular Ca^{2+} caused by the antagonizing effect of intracellular Mn^{2+} on Ca^{2+} extrusion and/or Ca^{2+} sequestration to the sarcoplasmic reticulum (Ushijima and Gomi, 1991). The present data show that the responses to oxytocin and neurokinin A could be reproduced at 60- to 100-min intervals in uterine strips maintained in Ca^{2+}-free, Mn^{2+}-containing solution with nifedipine in the presence or absence of cyclopiazonic acid throughout the experiment. This suggests that Mn^{2+} acts mainly by directly activating the contractile machinery, which is apparently very sensitive to Mn^{2+} in rat myometrium (Savineau et al., 1988).

In conclusion, our data indicate that Mn^{2+} acts as an antagonist of Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels in the rat uterus. In addition, Mn^{2+} ions penetrate the plasmalemma of uterine smooth muscle cells via voltage-operated Ca^{2+} channels but mainly via receptor-operated Ca^{2+} channels and produce contraction by directly activating the contractile machinery, but they are not able to substitute for Ca^{2+} in the Na^{+}/Ca^{2+} exchange mechanism or in the filling of internal Ca^{2+} stores. Hence, in the presence of a L-type Ca^{2+} channel blocker, Mn^{2+} could provide a useful tool for investigating the role played by receptor-operated Ca^{2+} channels in rat myometrial contractility.

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