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Oxytocin pretreatment of pregnant rat uterus inhibits Ca^{2+} uptake in plasma membrane and sarcoplasmic reticulum

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Myometrium from rats in varying stages of pregnancy (from 17 to 22 days) was treated with oxytocin (0.1–10 μM) and plasma membranes and sarcoplasmic reticulum were isolated using a Percoll gradient. When the myometrium had been treated with oxytocin, Ca^{2+} uptake was reduced by 29.4% in the plasma membrane and by 32.6% in the sarcoplasmic reticulum. The inhibitory action of oxytocin was highly dependent upon the stage of gestation: Only membranes from rats close to term (21–22 days) exhibited reduced Ca^{2+} transport activity after hormone treatment. This effect correlated highly with a significant decrease in the serum progesterone level of these animals. In plasma membrane vesicles, oxytocin reduced the maximal velocity of the Ca^{2+} pump without significantly affecting the affinity for Ca^{2+}. Oxytocin did not affect the passive permeability of the plasma membranes, nor their proportion of sealed inside-out vesicles nor the amount of Ca^{2+}-pump protein in these membranes. In addition, oxytocin caused no change in the passive permeability of the membrane nor in the rate of inositol trisphosphate-induced Ca^{2+} release from the sarcoplasmic reticulum. These results suggest that there is a specific action of oxytocin on the activity of the myometrial plasma membrane and sarcoplasmic reticulum Ca^{2+} pumps which may contribute to the maintenance of an elevated intracellular calcium level during parturition.

Introduction

The definition of the sources and routes of Ca^{2+} mobilization of the myometrium of the uterus is especially important because of the central role of Ca^{2+} in uterine contraction. The polypeptide hormone oxytocin is the agent which induces strong contractions of the uterus muscle at term, by increasing the cytoplasmic Ca^{2+} concentration [1]. The action of oxytocin on the Ca^{2+} mobilization is not yet completely described but the following steps involving it have been established. (1) It acts by binding to specific receptors on the cell surface. For this binding to cause contraction of the uterus, a coordinated increase in the number of receptor sites and the concentration of oxytocin must occur along with a decrease in the concentration of progesterone [2–4]. (2) Oxytocin acts by activation of phospholipase C to hydrolyze phosphatidylinositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG) through a GTP binding protein [5–9]. (3) Oxytocin enhances calcium influx into the cells, probably by opening receptor mediated Ca^{2+} channels [8,10]. (4) Oxytocin-generated IP_3 induces Ca^{2+} release from internal (non-mitochondrial) Ca stores [8]. Added IP_3 induced Ca^{2+} release from the sarcoplasmic reticulum of pregnant cow myometrium [11] and added IP_3 also induced contraction in skinned fibers of pregnant rat myometrium [12,13]. The present study aims at confirming another aspect of oxytocin action, its inhibition of Ca^{2+} pumps which remove Ca^{2+} from the cytosol. Removal of Ca^{2+} by its extrusion into the extracellular space can occur through two independent pathways, the Ca^{2+} pump and the Na^+/Ca^{2+} exchanger. Enyedi et al. [14] have observed that the Na^+/Ca^{2+} exchanger is much less efficient in myometrium than in brain or cardiac tissue. The effect of external sodium on oxytocin-induced Ca^{2+} fluxes was ruled out by Anwer and Sanborn [15] in isolated myometrium cells. These findings point to the importance of the plasma membrane Ca^{2+} pump as a target of oxytocin action. Inhibition of myometrial Ca^{2+}-ATPase by oxytocin was demonstrated by Akerman and Wikstrom [16], Soloff and Sweet [17] and Popescu

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et al. [18], but the Ca^{2+}-ATPase of myometrial membranes is primarily due to enzymes other than the Ca^{2+} pump [14]. Inhibition of Ca^{2+} binding by oxytocin was described in an unfractionated microsomal fraction from bovine [19] and human [20] uterus. Missiaen et al. [21], reported in passing that mixed microsomal preparations from pregnant rat myometrium showed partially inhibited Ca^{2+} uptake as a consequence of oxytocin treatment. However, they did not show any experimental data connected with this inhibition. A recent report of the effect of oxytocin on active Ca^{2+} transport was made by Enyedi et al. [22]. They used diethylstilbesterol-treated rats, and found that oxytocin inhibited Ca^{2+} uptake into myometrial plasma membranes by reducing the Ca affinity of the pump. Little attempt has yet been made to estimate the oxytocin-effect on Ca^{2+} re-uptake into the internal stores. Most of the above mentioned studies were carried out in uterine smooth muscle exposed to artificially imposed endocrine environments similar to those found during pregnancy (progesterone-dominated) and parturition (estrogen-dominated). In the current investigation, we examined the oxytocin effect on the Ca^{2+} transport of well-characterized plasma membrane and sarcoplasmic reticulum fractions of rat myometrium in different stages of actual pregnancy.

Materials and Methods

Materials

Sperm positive rats in different stages of gestation were obtained from the Holtzmann Company (Madison, WI, U.S.A.) and from BioLab (St. Paul, MN, U.S.A.). Percoll was obtained from Pharmacia and other chemicals used in the assay were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Oxytocin was obtained from Peninsula Laboratories, (Belmont, CA, U.S.A.). Oxytocin was freshly dissolved in 10 mM Tris-HCl (pH 7.2) before use. [γ-^{32}P]ATP, and $^{45}CaCl_2$ and [^{3}H]progesterone were obtained from New England Nuclear (Boston, MA, U.S.A.). Biotinylated anti-mouse IgG, and Vectastin ABC KIT were products of Vector Laboratories (Burlingame, CA, U.S.A.).

Preparation of plasma membranes

Two to four pregnant rats in the desired stages of gestation were killed by CO_2 chamber. Blood samples for progesterone radioimmunoassay were collected by heart puncture. The uterus horns were excised and trimmed of connective tissue and the fetuses and placentas were removed. The endometrium was gently scraped out of the uterus and the horns were washed four times with ice cold homogenization buffer containing 100 mM KCl/5 mM $MgCl_2$/10 mM Tris-HCl (pH 7.2) and 5 mM benzamidine, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 50 μg/ml trypsin inhibitor, 1 μg/ml leupeptin and 5 μg/ml aprotinin. The myometrium was minced with scissors and gently homogenized first in a Duall tissue grinder, then in a Potter-Elvehjem homogenizer. Membrane fractions were prepared as described by Enyedi et al. [22], with minor changes: all of the buffers used in the preparation contained the above mentioned amount of leupeptin and aprotinin, and the Percoll solution to form the self-generated Percoll gradient consisted of 18% Percoll. The final pellet was resuspended in 0.25 M sucrose in 10 mM Tris-HCl (pH 7.2) and was placed in small batches in liquid N_2. (Membranes stored under these conditions could be kept for months without losing their Ca^{2+} transport activity.) When both oxytocin treated and untreated (normal) membrane fractions were needed, the two horns of each uterus were separated and the two groups of muscle tissue were incubated with different amounts of oxytocin (zero and 0.1 or 10 μM) at 37°C for 10 min in the homogenization buffer. Both groups of tissues were then rapidly cooled to 4°C and subjected to additional procedures to prepare the plasma membrane and sarcoplasmic reticulum. All buffers used for the preparation of oxytocin-treated membranes contained 0.1 to 1 μM oxytocin, respectively. Protein was measured by the method of Lowry et al. [23].

Ca^{2+} uptake assay

Membranes (10–20 μg) were suspended in a total volume of 0.5 ml in an incubation medium containing 0.25 M sucrose, 50 mM Tris-HCl (pH 7.2), 0.1 mM ouabain, 2 μg/ml oligomycin, 10 mM $MgCl_2$, 6 mM ATP $\pm$ 5 mM potassium oxalate and 10 μM $CaCl_2$ (labelled with $^{45}Ca^{2+}$) and for the studies of activity as a function of Ca^{2+}, enough EGTA to produce the desired free Ca^{2+} concentration. The Ca^{2+} uptake at different incubation times was measured at 37°C by rapid filtration methods.

Ca^{2+} release assay

The assay medium contained 2 mM ATP, 3 mM $MgCl_2$, 160 mM KCl, 10 μM $CaCl_2$ (labelled with ^{45}Ca) and 20 mM K-phosphate buffer (pH 7.2). The reaction was started by 30–50 μg sarcoplasmic reticulum membrane vesicles, which were actively loaded with Ca^{2+} for 8 min at 37°C. EGTA was then added (2 mM) to reduce the free Ca^{2+} content below 0.1 nM. The buffer or different amounts of IP_3 or 0.2 μM A23187 (Ca^{2+} ionophore) were added to the sample 9 min after the Ca^{2+} had been added. Aliquots were taken (from the beginning of the assay) at 5, 8, 9.5, 10.5, 12, and 15 min. The Ca^{2+} content of the vesicles was established by rapid filtration methods [24].

Enzyme assays

5′-Nucleotidase and rotenone-insensitive NADH–cytochrome-*c* reductase were assayed by published proce-

dures [25]. ATPase activity was measured by monitoring the values of inorganic ^{32}P from [γ-^{32}P]ATP as described by Verma and Penniston [26] in a medium containing 3 mM ATP and 2 mM $CaCl_2$ or 2 mM $MgCl_2$. K^+-activated *p*-nitrophenylphosphatase activity was measured as described by Grover et al. [27]. The assay medium contained 5 mM $MgCl_2$, 5 mM KCl, ±1 mM ouabain and 0.25 M imidazole-HCl buffer (pH 7.8). Membranes (100 μg) were preincubated with ±0.1 μg digitonin/mg protein at room temperature for 20 min.

Cholesterol and progesterone assays

The total cholesterol concentration of the membrane fractions was determined with Sigma Diagnostic's Cholesterol 20 Kit. The serum progesterone level of pregnant rats was determined by radioimmunoassay. The progesterone antibody used had been raised against 6β-OH-progesterone, and was a gift from Dr. R.J. Ryan, Mayo Clinic. The antigen-antibody reaction was carried out with 50 μl serum samples in 40 mM Tris-HCl (pH 7.4), 0.5% bovine serum albumin, in the presence of 10000 cpm [^{3}H]progesterone overnight at room temperature. The rabbit progesterone antibody was diluted 1:2000. Three duplicates for each point were taken and the unspecific binding was subtracted out.

Preparation of Western blot

The monoclonal antibody 5F10 was used; this antibody was described by Borke et al. [28]. 1–20 μg of membrane proteins were separated by SDS-polyacrylamide gel electrophoresis on 0.75 mm thick 7.5% gels as described by Laemmli [29], using Pyronin Y as a tracking dye. Proteins were transferred to Millipore Immobilon PVDF transfer membrane as described by Towbin et al. [30], using 250 mA and 100V for 1 h. Immediately after the transfer the PVDF membrane was immersed in phosphate-buffered saline (PBS) containing 5% bovine serum albumin. The membrane was treated with 5F10 antibodies by the method of Burnette [31]. The staining was carried out with biotinylated antimouse IgG and avidin-horseradish peroxidase conjugate (Vectastain ABC KIT) which formed a colored product when diaminobenzamidine tetrahydrochloride plus H_2O_2 were added.

Results

The fractionation of pregnant rat myometrial membranes by Percoll density-gradient centrifugation is shown in Fig. 1. The plasma membrane markers 5'-nucleotidase and cholesterol have high values in the fractions close to the top of the gradient and are much lower or zero in the middle and bottom sections of the gradient. In the lower parts of the gradient, the sarcoplasmic reticulum marker enzyme rotenone-insensitive

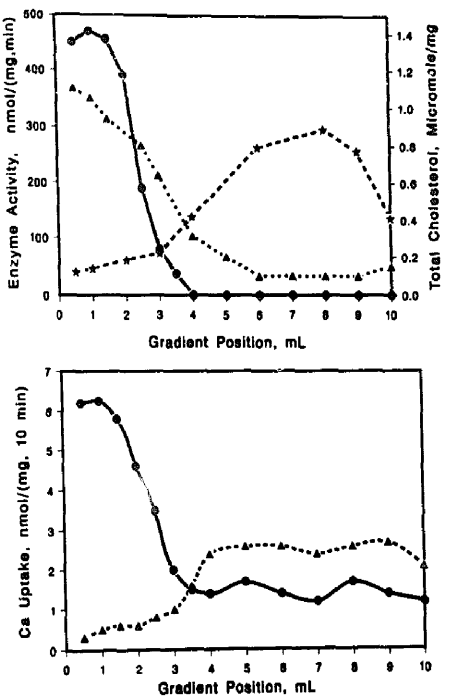

Fig. 1. Marker enzyme activities, cholesterol concentration and Ca^{2+} uptake in membrane fractions, fractionated on a Percoll gradient. Panel A: 5'-Nucleotidase (circles), and rotenone-insensitive NADH-cytochrome-*c* reductase (stars) activities are expressed as nmol of substrate/min per mg of protein. Total cholesterol concentration (triangles) is given as μmol per mg protein. Panel B: ATP-dependent Ca^{2+} uptake of the membrane fractions measured for 10 min at 37°C in an incubation medium containing 10 μM Ca^{2+} (labelled with ^{45}Ca) ±5 mM potassium oxalate, and expressed as nmol Ca^{2+}/10 min per mg of protein. The circles represent the Ca^{2+} uptake measured in the absence of oxalate anions, while the triangles show the increment in Ca^{2+} uptake due to addition of 5 mM oxalate. Membrane preparations were carried out without SH-group protection.

NADH-cytochrome-*c* reductase appears. In parallel Ca^{2+} transport experiments (Fig. 1B), the oxalate-independent Ca uptake coincided with the presence of the plasma membrane markers, while a lower degree of Ca^{2+} uptake, but a significantly oxalate-dependent one, was observed in the lower parts of the gradient. The oxalate-dependency of these lower fractions was highly dependent on the circumstances of membrane preparation. The presence of the SH-protecting agent, dithiothreitol (DTT) (in 0.1–1 mM concentration) enhanced the oxalate-dependent activity over 300% while the basal activity increased only 50–100%. On the other hand, pretreatment of the microsomal fraction with 0.6 M KCl to extract the contractile proteins from the membrane [32] had the opposite effect. The basal Ca^{2+} uptake was reduced drastically and the oxalate-depen-

dent Ca uptake was practically eliminated, even in the presence of 0.1 mM DTT. This kind of damage may be the reason for the observation by others of only a slight oxalate dependence of the Ca uptake activities of microsomal and SR fractions [33]. Due to the presence of one essential disulfide bridge in oxytocin, the effects of oxytocin must be observed without SH group protection. The relatively low Ca^{2+} uptake found in the sarcoplasmic reticulum (Table IB) is in good agreement with the results obtained from the same kind of pregnant rat sarcoplasmic reticulum by Missiaen et al. [34].

In the myometrial plasma membrane of pregnant rats, the detection of the Ca^{2+}-dependent ATPase activity of the pump proved to be impossible. The pump requires the presence of millimolar Mg^{2+} for its activity, and this much Mg^{2+} caused a very high non-pump Mg^{2+}-ATPase activity (3.4 ± 0.3 μmol/mg per min). The Ca^{2+}-ATPase activity, measured in the absence of Mg^{2+}, was similar to the Mg^{2+}-ATPase activity (3.1 ± 0.2 μmol/mg per min), could not be activated by calmodulin, and had a low affinity for Ca^{2+} (K_m 0.5 mM). This non-pump Ca^{2+}/Mg^{2+}-ATPase activity was 4–5-fold higher than was found by Enyedi et al. [14] in non-pregnant myometrial plasma membranes and was stable for months when the membranes were kept under liquid N_2. In the following experiments, the effect of different amounts of oxytocin on the ATP and Mg^{2+}-dependent Ca^{2+} uptake of plasma membrane vesicles and sarcoplasmic reticulum was studied. To exclude the possibility of any involvement of an ATP-dependent mitochondrial Ca^{2+} uptake, the reaction medium contained 2.0 μg/ml oligomycin.

It should be emphasized that the myometrial tissue was exposed to the hormone before its homogenization. The uterine horns were incubated with oxytocin (0.1 or 10 μM) at 37°C for 10 min. When oxytocin (0.01 to 10 μM) was added directly to membranes which had been isolated without oxytocin, no inhibitory effect was seen (data not shown). This result was expected, since the vesicles must be sealed and inside-out to accumulate Ca^{2+} and in such vesicles the oxytocin receptors are inaccessible. The data from 35 independent experiments on oxytocin inhibition are summarized in Tables 1A and B. As these tables show, 0.1 μM oxytocin gave significant inhibition of the Ca^{2+} uptake both in plasma membrane and in sarcoplasmic reticulum from rats in the last (22nd) day of pregnancy. On the 21st day, 0.1 μM oxytocin had no effect, but 10 μM oxytocin still caused inhibition. At earlier times during the gestation period (under 21 days), even 10 μM oxytocin caused no inhibition. Fig. 2 shows that serum progesterone levels were significantly reduced in the 21st and 22nd days of pregnancy. This finding is in good agreement with those of other laboratories [2,3].

A comparative study of Ca^{2+} transport by normal and oxytocin-treated membranes as a function of free Ca^{2+} concentration is shown in Fig. 3. The endogenous bound calmodulin was removed from the normal and oxytocin-treated membranes by: (1) incubation of the vesicles in 0.2 mM EGTA at 37°C for 5 min, and (2)

TABLE I

Correlation between the oxytocin effect and the stage of gestation on Ca^{2+} uptake of membrane vesicles prepared from pregnant rat uterus

Sperm positive day is day 'zero' with animals supposed to deliver on the 22nd day at dusk. Pregnant rats were killed at 2 p.m. on the 22nd day of pregnancy, or at 8 a.m. on the other days. Calcium uptake was measured (at 10 μM total $CaCl_2$ and no EGTA or added calmodulin) in membrane vesicles treated with different amounts of oxytocin as described in Materials and Methods.

Day of pregnancy	Concn. of oxytocin (μM)	Number of experiments	Number of effective inhibitions	Average of Ca^{2+} uptake (nmol/mg per 5 min)	Average of inhibition (%)
A. Oxytocin effect on Ca^{2+} uptake by plasma membrane vesicles					
22	10	13	11	2.53±0.41	29.4±8.03
22	0.1	4	3	2.45±0.52	26.1±5.1
21	10	7	3	3.05±1.08	23.7±4.78
21	0.1	5	0	3.2 ±1.03	0
20	10	3	0	3.2 ±0.6	0
19	10	3	0	3.2 ±0.4	0
17	10	2	0	3.15±1.0	0
B. Oxytocin effect on Ca^{2+} uptake by sarcoplasmic reticulum, measured in the presence of 5 mM oxalate					
22	10	13	10	2.0 ±0.72	32.6±5.4
22	0.1	4	1	1.8	27.1
21	10	7	3	2.2 ±0.61	29.7±4.3
21	0.1	5	0	2.0 ±0.47	0
20	10	3	0	2.2 ±0.54	0
19	10	3	0	1.9 ±0.53	0
17	10	2	0	1.7 ±0.41	0

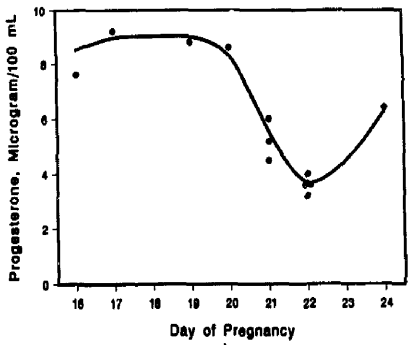

Fig. 2. Correlation between the serum progesterone level and the stage of gestation. The radioimmunoassay from 50 μl rat serum was carried out as described in Materials and Methods. Blood samples were collected by heart puncture, and centrifuged at 3000 rpm for 5 min. The sera from different animals in the same experiments were mixed in equal ratios and stored at −20°C until measuring.

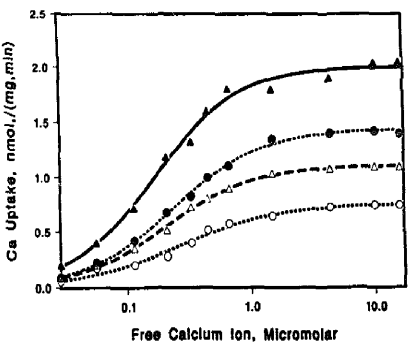

Fig. 3. ATP-dependent Ca^{2+} uptake with and without oxytocin as a function of free Ca^{2+} concentration. Membrane vesicles from 22-day pregnant rats at a concentration of 1.5 mg protein/ml were preincubated at 37°C for 5 min in the presence of 0.2 mM EGTA. 10 μl of this membrane suspension was transferred to the transport medium containing the desired free Ca^{2+} concentration, and after three minutes incubation at 37°C the reaction was started with ATP and incubated a further 5 min before stopping by filtration. Triangles represent Ca^{2+} uptake by normal membranes, while circles represent Ca^{2+} uptake by oxytocin-treated membranes. Filled symbols show the Ca^{2+} uptake in the presence of 20 μg calmodulin/ml, unfilled symbols show the Ca^{2+} uptake in the absence of calmodulin.

addition of the synthetic calmodulin binding peptide, C28W in a 0.5 μM concentration to the Ca^{2+} uptake medium. These treatments were used previously to remove the calmodulin from non-pregnant rat myometrial plasma membrane and proved adequate in that case [22]. Maximal inhibition (up to 40%) was observed already at a low (0.1 μM) free Ca^{2+} concentration and was maintained at higher Ca^{2+} concentrations. During this reduction of V_{max}, no significant change in $K_{1/2}$ for Ca^{2+} activation was detectable either in the presence or in the absence of calmodulin (Fig. 3). Treating the tissue with less oxytocin (0.1 μM) caused the same changes in the Ca^{2+} kinetics as did more oxytocin, both in the absence of calmodulin (Fig. 4) and in its presence (data not shown). Data from the kinetic analysis of a number of independent Ca uptake experiments are summarized in Table II.

Experiments were carried out to check whether oxytocin treatment caused any change in the membrane permeability to Ca^{2+} or in the membrane sidedness. The membrane permeability to Ca^{2+} was monitored in the following manner: After 5 min of Ca^{2+} uptake, the extravesicular Ca^{2+} was reduced to a negligible level by addition of EGTA, and the rate of Ca^{2+} release was monitored. Fig. 5 shows that the rate of Ca^{2+} release from loaded vesicles was similar in untreated and in hormone-treated membrane vesicles in both the plasma membrane (Fig. 5A) and sarcoplasmic reticulum (Fig. 5B) preparations. The sidedness of the membranes was assessed by measuring the ouabain-sensitive K^+-activated *p*-nitrophenylphosphatase activity in the presence and absence of digitonin. This activity is due to the Na^+/K^+ pump; in the absence of digitonin, ouabain will bind to (and inhibit) the pump molecules *only* in right-side-out or leaky vesicles. In the presence of dig-

TABLE II

Kinetic parameters of active Ca^{2+} transport in normal and oxytocin-treated plasma membranes

The values of V_{max} and $K_{1/2}$ for Ca^{2+} activation were estimated from the Hill plots. The incubation times for the assays were 5 min, and the membranes were derived from rats in the 22nd day of pregnancy.

Treatment	− Calmodulin		+ Calmodulin	
	V_{max} (nmol/mg per min)	$K_{1/2}$, Ca^{2+} (μM)	V_{max} (nmol/mg per min)	$K_{1/2}$, Ca^{2+} (μM)
Untreated plasma membrane	1.06 ± 0.15 (9)	0.29 ± 0.07 (9)	1.89 ± 0.15 (6)	0.20 ± 0.04 (6)
Oxytocin (10 μM) treated plasma membrane	0.73 ± 0.16 (7)	0.32 ± 0.07 (7)	1.27 ± 0.16 (6)	0.23 ± 0.02 (6)
P	0.0011	0.59	0.00007	0.14

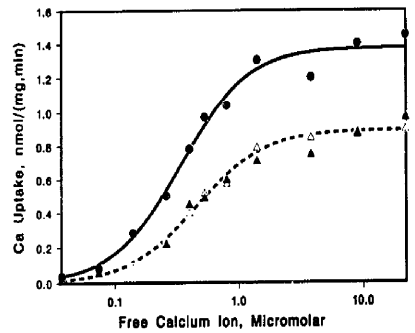

Fig. 4. Ca^{2+} uptake in plasma membrane vesicles treated with different amounts of oxytocin. The assay was performed as described in the legend of Fig. 3. The Ca^{2+} uptake was measured in the absence of calmodulin. The circles represent the Ca^{2+} uptake by normal vesicles, while the triangles represent oxytocin-treated membrane vesicles. Before the membrane preparation the myometrial tissue was preincubated with 10 μM oxytocin (open triangles) or with 0.1 μM oxytocin (filled triangles).

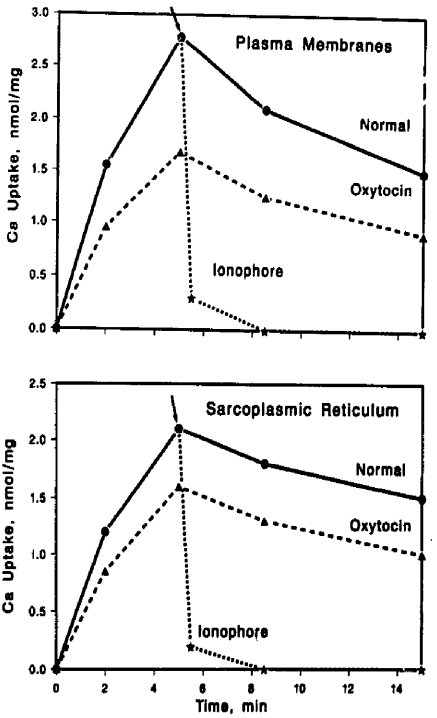

Fig. 5. Passive Ca^{2+} leak by oxytocin-treated (10 μM) and untreated plasma membrane vesicles and sarcoplasmic reticulum. The membranes were preincubated for 5 min at 37°C in the presence of 6 mM ATP, 5 mM $MgCl_2$ and 10 μM $CaCl_2$. After 5 min 5 mM EGTA was added to reduce the Ca^{2+} concentration in the medium. The solid lines with filled circles represent the untreated, while the dashed lines with filled triangles represent the oxytocin-treated membranes. The dotted lines with stars show the Ca^{2+} release from the membrane vesicles induced by 0.2 μM A23187 calcium ionophore.

itonin, ouabain will bind to all the pump molecules, so that the proportion of right-side-out vesicles can be estimated from these two activities. As is shown in Table III, no change in the proportion of right-side-out vesicles was caused by oxytocin treatment. In both of the membranes, the estimated proportion of inside-out vesicles was 57%.

Immunoblots of untreated and oxytocin-treated myometrial plasma membrane proteins and human erythrocyte ghosts can be seen in Fig. 6. These blots were done using monoclonal antibody 5F10, which was raised against the purified erythrocyte Ca^{2+} pump and cross-reacts with plasma membrane Ca^{2+} pumps from many species and tissues [28]. Treatment of the myometrium with oxytocin did not cause any changes in the immunoreactivity of the plasma membranes. Three different protein concentrations (1, 5, and 10 μg/track) led to the same conclusion (data not shown), which indicates the presence of the same amount of pump protein in untreated and oxytocin-treated plasma membranes.

The sarcoplasmic reticulum of the myometrium exhibits IP_3-sensitive Ca^{2+} ion channels. To investigate

TABLE III

Study on vesicle orientation of normal and oxytocin-treated plasma membranes

K^+-activated ouabain-sensitive and -insensitive *p*-nitrophenylphosphatase (PNPPase) activities were measured as described in Materials and Methods. The latent activities were determined in the absence, the total activities in the presence of 0.1 μg/ml digitonin. The ouabain sensitive activity was that part of the PNPPase that was inhibited by 1 mM ouabain. The latent PNPPase activity was corrected for the increase in total PNPPase activity caused by digitonin.

PNPPase component	PNPP hydrolyzed (μmol/mg per h)			
	plasma membrane normal		plasma membrane oxytocin-treated (10 μM)	
	latent	total	latent	total
K^+-activated ouabain-sensitive	0.201	0.48	0.138	0.37

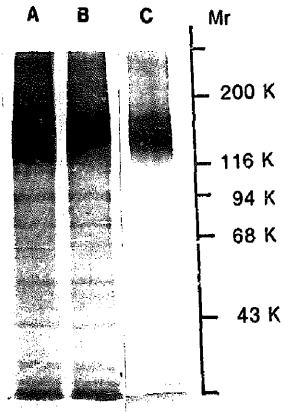

Fig. 6. Western blot of normal and oxytocin (10 μM) treated myometrial plasma membrane proteins. The monoclonal antibody (5F10) was raised against purified human erythrocyte Ca^{2+} pump. Lane A: 15 μg/lane untreated plasma membrane proteins. Lane B: 15 μg/lane oxytocin treated plasma membrane proteins. Lane C: 15 μg/lane erythrocyte inside-out vesicle proteins.

possible alterations of these channels during the oxytocin treatment, experiments were carried out comparing the IP_3-induced Ca^{2+} release in the two different sarcoplasmic reticulum preparations. Data cited in Table IV give evidence that neither the degree of maximal release (approx. 23%) nor the sensitivity to IP_3 were changed by oxytocin treatment of the membranes.

Discussion

Our results indicate a significant inhibition by oxytocin of both the plasma membrane and sarcoplasmic reticulum Ca^{2+} pumps. This inhibition can be readily detected by measuring the effect of oxytocin on Ca^{2+} uptake, but not by measuring Ca^{2+}-ATPase. Ca^{2+}-ATPase is not an indicator of pump activity because a very high activity (over 3 μmol/mg per min) Ca^{2+}- or Mg^{2+}-ATPase in the plasma membranes hides the relatively small ATPase activity due to the Ca^{2+} pump. In order to assess the pump activity by measuring Ca^{2+} uptake, it was necessary to consider possible changes in membrane permeability or alteration in the fraction of closed inside out vesicles. Such changes would strongly influence the observed rate of Ca^{2+} uptake by the vesicles. In this paper, we showed that oxytocin caused no change in the passive permeability of the plasma membrane or in the proportion of inside out vesicles.

The results of a Western blot analysis reconfirmed the presence of the erythrocyte-type Ca^{2+} pump proteins in these plasma membranes, and showed that the quantity of pump protein was not affected by oxytocin. This indicates that the change in the amount of Ca^{2+} pumped must be due to a change in the specific activity of the pump presumably due to some regulatory modification of the pump or its surroundings.

Enyedi et al. [22], showed the inhibition of Ca^{2+} uptake by oxytocin in estradiol-treated myometrial plasma membrane vesicles. In those non-pregnant rats, oxytocin decreased the apparent affinity of the calcium pump for Ca^{2+} without affecting the maximal velocity of the pump. Conversely, in pregnant rat myometrial plasma membrane vesicles, the effect of the oxytocin on the Ca uptake is already maximal at low (0.1 μM) free Ca^{2+} concentrations, and the hormone reduces the maximal velocity for the Ca^{2+} pump both in the presence and absence of calmodulin without altering the affinity of the pump for Ca^{2+}. Other differences between pregnant and non-pregnant myometrial plasma membrane Ca^{2+} uptake can be noticed in the apparent affinity for Ca^{2+} in the absence of calmodulin, which is significantly higher in pregnant rats, and in the total activity, which is lower in pregnant rats. This altered response to Ca^{2+} cannot be explained by the inefficient removal of the endogenous calmodulin from the membrane, because the addition of calmodulin induced a similar increase in the maximal velocity of Ca^{2+} uptake in both pregnant and non-pregnant plasma membrane vesicles. It may be that the altered response is due to a change in the isozyme composition of the Ca^{2+} pump or to a change in the lipid composition or fluidity of the membrane.

In vivo, the myometrial response to oxytocin is regulated by the oxytocin receptor level as well as by the circulating oxytocin concentration. The concentration of oxytocin receptors and their sensitivity to the hormone is significantly enhanced at the end of pregnancy [35]. The appearance of the oxytocin receptors is,

TABLE IV

IP_3 induced Ca^{2+} release from actively loaded sarcoplasmic reticulum

Ca^{2+} release induced by various amounts of IP_3 was measured in the presence of 20 μM phosphate anion, 3 mM $MgCl_2$ and 2 mM ATP, as it is described in Materials and Methods. During the loading of the vesicles, the Ca^{2+} concentration was 10 μM, and it was decreased to less than 0.1 nM by EGTA before starting the Ca^{2+} release by IP_3. Similar experiments were carried out with 0.2 μM A23187 Ca^{2+} ionophore to determine the releasable intracellular Ca^{2+} content.

Membranes	Concentration of IP_3 (μM)	Percent of intravesicular Ca^{2+} content released
Normal sarcoplasmic reticulum	0	0
	7	6
	12	19
	14	21
	24	23
Oxytocin (10 μM) treated sarcoplasmic reticulum	0	0
	7	8
	14	24

in turn, regulated by the decrease of progesterone in the serum and in the tissue [36]. The strong correlation between the oxytocin effect and the serum progesterone level in our experiments reconfirmed the suggestion that the effects of oxytocin on the Ca^{2+}-transport system, like other receptor-mediated oxytocin effects, are antagonized by progesterone. In order to assure access of oxytocin to its receptors, it was necessary to pretreat the tissue with oxytocin and prepare the vesicles in a medium containing oxytocin. Since the membrane isolation procedure may alter the oxytocin concentration in the vesicles, we routinely used a high (10 μM) oxytocin concentration, which gave consistent results in 21- and 22-day pregnant rats. Similar inhibition of Ca^{2+} uptake could be obtained at a much lower oxytocin concentration (0.1 μM) in myometrial plasma membrane vesicles from rats which were very close to term. The strong relationship between the stage of pregnancy and the inhibitory effect of oxytocin in both the plasma membrane and the sarcoplasmic reticulum confirm the specificity of these hormone actions on the Ca uptake.

Based on the enzyme marker distribution and on Western blot analysis (not shown), the contamination of the sarcoplasmic reticulum with plasma membrane is less than 20–25%, so that effects of oxytocin on the sarcoplasmic reticulum cannot result from plasma membrane contamination. Because the sarcoplasmic reticulum does not contain oxytocin receptors an irreversible modification of the pump protein or the modification of the surrounding membrane has to be proposed to explain oxytocin's effect on sarcoplasmic reticulum.

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