Oxytocin regulates the plasma membrane Ca\textsuperscript{2+} transport in rat myometrium

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INTRODUCTION

It is widely accepted that oxytocin is the most potent of all agents in inducing strong contractions of the uterine smooth muscle at term, and that its action results in an elevated intracellular free [Ca\textsuperscript{2+}] (Huszar, 1986). Although the details of the molecular mechanism of oxytocin action are not yet known, it is generally assumed that it has its effect by: (1) opening Ca\textsuperscript{2+} channels and/or (2) by liberating a signal molecule, which then releases Ca\textsuperscript{2+} from internal stores; and (3) by inhibiting of mechanisms responsible for Ca\textsuperscript{2+} extrusion from the cell. The aim of the present work was to examine this last possibility. Several laboratories have reported the inhibition by oxytocin of activities they believed to be associated with the plasma membrane Ca\textsuperscript{2+} pump (Akerman & Wikstrom, 1979; Soloff & Sweet, 1982; Popescu et al., 1985; Huszar, 1986). However, none of these reports settled the issue, because all of them identified the Ca\textsuperscript{2+}-ATPase activity of the plasma membrane as the Ca\textsuperscript{2+} pump. We have shown (Enyedi et al., 1988) that a calmodulin-stimulated Ca\textsuperscript{2+}-transport mechanism is present in the myometrial plasma membrane and that this Ca\textsuperscript{2+} pump has quite different characteristics from the Ca\textsuperscript{2+}-ATPase of the same membrane. This Ca\textsuperscript{2+}-ATPase has a much higher activity than does the Ca\textsuperscript{2+} pump, so that the ATPase activity of the pump is not detectable in the presence of such a large amount of non-pump Ca\textsuperscript{2+}-ATPase. This indicates that the reports mentioned above are not directly relevant to the effect of oxytocin on Ca\textsuperscript{2+} transport.

Another laboratory (Carsten, 1974, 1979) reported that oxytocin inhibited ATP-dependent Ca\textsuperscript{2+} binding to microsomes from the myometrium. In these studies, the microsomal fraction was referred to as microsomes or sarcoplasmic reticulum, and the relationship of the Ca\textsuperscript{2+} binding to active Ca\textsuperscript{2+} transport was not explored.

In an effort to address this problem, we report here the effect of oxytocin on the active transport of Ca\textsuperscript{2+} in a well-defined plasma membrane preparation from myometrium and compare this with the effect of oxytocin on sarcoplasmic reticulum from the same tissue. In order to obtain a faster and more complete separation of plasma membrane from sarcoplasmic reticulum, we developed and used a new Percoll-gradient procedure for purification of the membranes.

MATERIALS AND METHODS

Materials

Immature female Holtzman rats, 22 days old, were obtained from the Holtzman Company, Madison, WI. U.S.A. \textsuperscript{45}CaCl\textsubscript{2} was obtained from New England Nuclear, Boston, MA, U.S.A. Diethylstilboestrol (DES), progesterone, Percoll and other chemicals used in the assays 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. DES was dissolved in ethanol at a concentration of 4 mg/ml. This stock solution was freshly diluted into iso-osmotic saline at 60–70 °C with vigorous stirring to obtain a clear solution of 0.04 mg of DES/ml of saline containing 1% ethanol. Then the solution was cooled down to about 37 °C before use. In experiments where progesterone was needed, the hormones were dissolved in sesame oil.
Separation of plasma membrane and sarcoplasmic reticulum by Percoll density-gradient centrifugation

Thirty rats were injected intraperitoneally with 10 μg of DES in 0.25 ml of saline containing 1% ethanol daily for 3 days (unless otherwise noted) and were killed on the fourth day. In some experiments rats were divided into two separate groups: 15 rats were injected with 10 μg of DES in 0.25 ml of sesame oil and the rest were injected with 10 μg of DES plus 0.33 mg of progesterone in 0.25 ml of sesame oil, on 3 successive days. The uterine horns were excised and trimmed of connective tissue, fat, and endometrium. The myometrium was minced with a scissors and gently homogenized in a Duall tissue grinder and then with a Potter–Elvehjem homogenizer in 100 mM-KCl/5 mM-MgCl2/50 mM-Tris/HCl, pH 7.2, at 0 °C as described previously (Enyedi et al., 1988). The homogenate was centrifuged (Beckman JA20 rotor, 12000 rev./min) at 20000 g for 10 min to remove nuclei, mitochondria and fibrous material. The supernatant was centrifuged at 160000 g (Ti50 rotor, 45000 rev./min) for 30 min. The pellet was briefly homogenized in 6 ml of buffer containing 0.25 M-sucrose, 5 mM-MgCl2 and 10 mM-Tris/HCl, pH 7.2. A volume of this suspension (1.5 ml) was layered on to 10 ml of 20% Percoll in the pellet homogenization buffer in a 15 ml Corex tube. Four tubes were centrifuged in a Beckman JA20 rotor at 17000 rev./min (maximum force 33000 g) for 30 min. Then, 1 ml fractions were harvested from the top of the self-generated Percoll gradient using a pipette; the corresponding fractions from the four tubes were always pooled. Since Percoll did not interfere with enzymic assays, marker enzymes of the plasma membrane and sarcoplasmic reticulum were measured immediately. After the procedure was characterized, fractions 1 and 2 of the gradient (corresponding to the upper band) were pooled as plasma membrane. The sarcoplasmic reticulum usually appeared as two separate bands, SRI and SRII. Fractions 5 and 6 were pooled (SRI) and 7 and 8 formed a separate pool (SRII). For measurements of Ca2+ uptake, the pooled fractions were centrifuged at 160000 g for 1 h to remove Percoll. The Percoll beads were pelleted at the bottom of the tubes, and the membranes formed a layer on the surface of the Percoll pellet. The membrane layer was harvested and resuspended thoroughly in 0.25 M-sucrose/10 mM-Tris/HCl, pH 7.2. Normally thirty rats were used, giving about 3-4 mg of plasma membrane and 2-3 mg of SR protein. All buffers used for the preparation of membrane contained 5 mM-benzamidine, 0.5 mM-phenylmethylsulphonyl fluoride and trypsin inhibitor (50 μg/ml), except that phenylmethylsulphonyl fluoride and trypsin inhibitor were omitted from the final solution.

Membranes prepared from oxytocin-treated muscle are called 'oxytocin-treated membranes' to distinguish them from 'normal membranes'. Where both kinds of membrane were needed, the two horns of each uterus were separated. One horn was used to prepare normal membranes and the other horn was used to prepare oxytocin-treated membranes. The myometria for the oxytocin-treated membranes were incubated with 10 μM-oxytocin in homogenization buffer at 37 °C for 15 min, while the myometria for normal membranes were similarly incubated without oxytocin. Both were then cooled to 4 °C and subjected to the procedure for preparation of plasma membranes and sarcoplasmic reticulum.

All buffers used for the preparation of oxytocin-treated membranes contained 1 μM-oxytocin. Protein was measured by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Ca2+ uptake assay

Ca2+ uptake by plasma membranes or sarcoplasmic reticulum vesicles was measured by rapid filtration through Millipore membrane filters (0.45 μm pore size, type HA) as described (Enyedi et al., 1988). Membranes (10–15 μg) were suspended to a total volume of 0.5 ml in the transport medium, which also contained 0.25 M-sucrose, 50 mM-Tris/HCl, pH 7.2, 0.1 mM-ouabain, 10 mM-MgCl2, 6 mM-ATP, ± 5 mM-potassium oxalate, 100 μM-CaCl2 (labelled with 45Ca) and enough EGTA to produce the desired free Ca2+ concentrations, and were incubated at 37 °C for 5 min unless otherwise noted. The incubation medium also contained 40 μg of calmodulin/ml or the calmodulin-binding peptide C28 (0.5 μM) where it was needed. This calmodulin-binding peptide has a very high affinity for calmodulin, and has the sequence LRRGQLWFRGLNRIQTQIKVVNASTSS, which is the sequence found in the calmodulin-binding region of the erythrocyte Ca2+ pump (James et al., 1989).

Enzyme assays

The following enzyme activities were assayed on a Beckman model 25 double beam spectrophotometer by published procedures: S'-nucleotidase, NADPH–cytochrome c reductase and rotenone-insensitive NADH–cytochrome c reductase (Kwan et al., 1979).

RESULTS

Fig. 1 shows the separation of the myometrial plasma membrane and sarcoplasmic reticulum by Percoll density-gradient centrifugation. As can be seen in Fig. 1(a), the plasma membrane marker enzyme S'-nucleotidase is highly enriched in the fractions close to the top of the gradient, and the sarcoplasmic reticulum marker enzyme rotenone-insensitive NADH–cytochrome c reductase appears almost exclusively in a relatively broad peak in the middle section of the gradient. In parallel experiments, active Ca2+ transport was measured in the presence and absence of 5 mM-oxalate (Fig. 1b). The oxalate-independent Ca2+ uptake coincided with the appearance of the plasma membrane marker, whereas highly oxalate-dependent Ca2+ transport could be observed where the sarcoplasmic reticulum marker enzyme activities were detected. In most cases, the membrane with the characteristics of the sarcoplasmic reticulum could be seen as two separate membrane fractions, but the particular experiment shown in Fig. 1 did not resolve these two sarcoplasmic reticulum fractions. A plasma membrane fraction and two separate sarcoplasmic reticulum fractions (SRI and SRII) were routinely collected. The distribution of marker enzymes among these fractions after removal of Percoll by high-speed centrifugation is shown in Table 1. A constant Mg2+-ATPase activity, which is inhibited by oxytocin, has been described in a crude microsomal preparation from rat myometrium (Missiaen et al., 1988). We show here that most of this Mg2+-ATPase activity appears in the plasma membrane fraction enriched in S'-nucleotidase activity. On the other hand, the SRI and SRII fractions contained the highest specific activities of
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Fig. 1. Enzyme distribution and Ca\textsuperscript{2+} uptake in membrane fractions fractionated on Percoll gradient

(a) 5'-Nucleotidase (○) and rotenone-insensitive NADH-cytochrome c reductase (△) activities expressed as nmol of substrate/min per mg of protein. (b) ATP-dependent Ca\textsuperscript{2+} uptake by 20 μl aliquots of membrane fractions measured for 20 min at 37°C in an incubation medium containing 6 μM-free Ca\textsuperscript{2+} ± 5 mM-potassium oxalate, and expressed as nmol of Ca\textsuperscript{2+}/20 min per mg of protein. ▲, Ca\textsuperscript{2+} uptake measured in the absence of oxalate; △, increment in Ca\textsuperscript{2+} uptake due to addition of 5 mM-oxalate.

NADH- and NADPH-cytochrome c reductases and almost exclusively the oxalate-stimulated Ca\textsuperscript{2+} uptake. Active Ca\textsuperscript{2+} transport in either the sarcoplasmic reticulum or plasma membrane fraction was not affected by oligomycin in a concentration (20 μg/mg of protein) high enough to completely inhibit Ca\textsuperscript{2+} transport in mitochondria (results not shown). Therefore, the possibility of the involvement of mitochondria can be excluded.

Fig. 2 shows the time-course of active Ca\textsuperscript{2+} transport by plasma membrane (a) and SRII (b) membrane fractions. The Ca\textsuperscript{2+} uptake by the plasma membrane vesicles was not dependent on the presence of oxalate, whereas the ATP-fuelled Ca\textsuperscript{2+} accumulation into the SRII fraction was substantially increased by this anion, as expected (Grover, 1985). The oxalate-sensitivity of Ca\textsuperscript{2+} uptake by sarcoplasmic reticulum was much higher after Percoll-gradient separation than it was after other methods using sucrose-density-gradient centrifugations (Grover & Kwan, 1983).

In the following experiments the effect of oxytocin on the ATP-Mg\textsuperscript{2+}-dependent Ca\textsuperscript{2+} uptake by plasma membrane vesicles was studied. Fig. 3 shows that oxytocin inhibited active Ca\textsuperscript{2+} transport by 20–25% at 1 μM-free Ca\textsuperscript{2+} in membranes derived from oxytocin-treated muscle. This effect was consistently observed when rats were treated intraperitoneally with 10 μg of DES for 3 days. The 3-day injection period resulted in uterus which were swollen with fluid and not quite fully developed. Further hormone administration somewhat increased the weight of the uterus, giving a more muscular, less bloated appearance, but it diminished the effect of oxytocin on Ca\textsuperscript{2+} transport. When rats were treated for 6 days with this amount of DES, inhibition by oxytocin was smaller and was difficult to observe consistently.

Fig. 3 also shows that oxytocin did not affect the passive Ca\textsuperscript{2+} leak by these vesicles. Ca\textsuperscript{2+} uptake was measured in the presence of ATP plus Mg\textsuperscript{2+}, and after incubation for 5 min 2 mM-EGTA was added. The rate of EGTA-dependent release of Ca\textsuperscript{2+} was identical in both normal and oxytocin-treated membrane preparations. It is worth mentioning that no effect of oxytocin was observed on membranes isolated from muscle which had not been pretreated with oxytocin. The most probable explanation for this is that Ca\textsuperscript{2+} uptake is measured in inside-out vesicles whereas oxytocin receptors are exposed only in right-side-out vesicles.

A comparative study of Ca\textsuperscript{2+} transport by normal and oxytocin-treated membranes as a function of free Ca\textsuperscript{2+} concentration is shown in Fig. 4. In order to exclude the possibility that the amount of calmodulin bound to the membranes was different, both types of membranes were preincubated with 0.2 mM-EGTA at 37°C for 5 min as

Table 1. Distribution of enzyme markers and oxalate-sensitive and -insensitive Ca\textsuperscript{2+} transport in Percoll-density-gradient fractions

<table>
<thead>
<tr>
<th>Membrane</th>
<th>5'-Nucleotidase</th>
<th>Rotenone-insensitive NADH-cytochrome c reductase</th>
<th>NADPH-cytochrome c reductase</th>
<th>Mg\textsuperscript{2+}-ATPase</th>
<th>Ca\textsuperscript{2+} uptake (nmol of Ca\textsuperscript{2+}/20 min per mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM</td>
<td>375</td>
<td>51</td>
<td>11.6</td>
<td>793</td>
<td>20.6</td>
</tr>
<tr>
<td>SRI</td>
<td>100</td>
<td>213</td>
<td>21.3</td>
<td>n.m.</td>
<td>13.3</td>
</tr>
<tr>
<td>SRII</td>
<td>0</td>
<td>265</td>
<td>27.8</td>
<td>278</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Marker enzyme activities were measured as described in the Materials and methods section. Ca\textsuperscript{2+} uptake was measured in an incubation medium containing 6 μM-free Ca\textsuperscript{2+} for 20 min, in the presence and absence of 5 mM-potassium oxalate. Mg\textsuperscript{2+}-ATPase activity was measured in a medium containing 100 mM-KCl, 1 mM-ATP (labelled with [γ\textsuperscript{32P}]ATP), 2 mM-MgCl\textsubscript{2}, 1 mM-EGTA, 0.1 mM-ouabain, 50 mM-Tes-triethanolamine, pH 7.2 at 37°C for 5 min by monitoring the release of inorganic [γ\textsuperscript{32P}]P, from [γ\textsuperscript{32P}]ATP as previously described (Verma & Penniston, 1981). PM, plasma membrane; n.m., not measured.
The incubation medium contained 6 μM-free Ca\(^{2+}\). ○, No addition; ●, plus 5 mM-potassium oxalate. (a) Plasma membrane, (b) sarcoplasmic reticulum, fraction II.

described (Enyedi et al., 1988). Fig. 4(a) shows the Ca\(^{2+}\)-concentration-dependence of Ca\(^{2+}\) transport in the absence of calmodulin (in order to inactivate any remaining calmodulin, the incubation medium also contained a 0.5 μM concentration of the synthetic calmodulin-binding peptide, C28). A maximal inhibition of 50-60% was observed at low (0.1-0.2 μM) free Ca\(^{2+}\) concentrations, but no inhibition by oxytocin was found at higher than a 1.5-2 μM-Ca\(^{2+}\) concentration. The \(K_{i_{ax}}\) for Ca\(^{2+}\) activation was twice as high in the oxytocin-treated membranes as in the normal ones, while the \(V_{max}\) did not change significantly (Table 2).

The effect of calmodulin on Ca\(^{2+}\) uptake by normal and oxytocin-treated membrane vesicles can be seen in Fig. 4(b). In both normal and oxytocin-treated membranes, Ca\(^{2+}\) transport was substantially activated
Table 2. Kinetic parameters of active Ca\(^{2+}\) transport in normal and oxytocin-treated plasma membranes (PM)

The values of \(V_{\text{max}}\) and \(K_{1/2}\) for Ca\(^{2+}\) activation were estimated from a Hill plot of the data shown in Fig. 4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(-) Calmodulin</th>
<th>+ Calmodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(V_{\text{max}}) (nmol/min per mg)</td>
<td>(K_{1/2}), Ca(^{2+}) ((\mu)M)</td>
</tr>
<tr>
<td>Normal PM</td>
<td>1.49 ± 0.046</td>
<td>0.41 ± 0.035</td>
</tr>
<tr>
<td>Oxytocin-treated PM</td>
<td>1.48 ± 0.067</td>
<td>0.93 ± 0.012</td>
</tr>
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Table 3. Ca\(^{2+}\) uptake by normal and oxytocin-treated plasma membrane vesicles derived from DES- and (DES + progesterone)-treated rats

One group of rats was treated with 10 \(\mu\)g of DES, the other group with 10 \(\mu\)g of DES + 0.33 \(\mu\)g of progesterone for 3 days as described in the Materials and methods section. Ca\(^{2+}\) uptake by normal and oxytocin-treated plasma membrane (PM) vesicles was measured at a calculated free Ca\(^{2+}\) concentration of 0.26 \(\mu\)M as described in the legend to Fig. 4. The data shown are means ± s.d. of Ca\(^{2+}\) uptake activities assayed in triplicate. The results of a typical experiment are shown. Similar results were obtained in two other experiments. CaM, calmodulin.

<table>
<thead>
<tr>
<th></th>
<th>DES-treated rats</th>
<th>DES + progesterone-treated rats</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(-) CaM</td>
<td>+ CaM</td>
</tr>
<tr>
<td>Normal PM</td>
<td>0.99 ± 0.050</td>
<td>2.15 ± 0.037</td>
</tr>
<tr>
<td>Oxytocin-treated PM</td>
<td>0.52 ± 0.037</td>
<td>1.72 ± 0.038</td>
</tr>
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</table>

by calmodulin; the \(V_{\text{max}}\) was almost doubled and the \(K_{1/2}\) for Ca\(^{2+}\) activation was shifted to lower values in both cases. Even in the presence of calmodulin the \(K_{1/2}\) for Ca\(^{2+}\) still remained higher in case of the oxytocin-treated membranes than in the normal ones. The maximal velocity was again unchanged by oxytocin treatment.

The physiological relevance of the effect of oxytocin on plasma membrane Ca\(^{2+}\) transport was studied by use of progesterone. It is known that progesterone prevents the oestrogen-induced increase in oxytocin receptors in rats (Fuchs, 1986). Therefore rats were divided into two groups: one group was treated with 10 \(\mu\)g of DES while the other was treated with the same amount of DES plus 330 \(\mu\)g of progesterone. Normal and oxytocin-treated membrane vesicles were isolated and active Ca\(^{2+}\) transport was measured at 0.26 \(\mu\)M-free Ca\(^{2+}\). Data from this experiment are shown in Table 3. No effect of oxytocin was found in membranes from rats treated with DES and progesterone together, but a 50% inhibition was observed in the absence and about 20% in the presence of calmodulin in the other group.

The effect of oxytocin treatment on the oxalate-stimulated Ca\(^{2+}\) transport of the sarcoplasmic reticulum was also studied. In half of the membrane preparations about 20% inhibition was observed at all Ca\(^{2+}\) concentrations examined, but no characteristic change in the \(K_{1/2}\) for Ca\(^{2+}\) activation was found (results not shown); in the remaining preparations, no effect of oxytocin was found.

**DISCUSSION**

Separation of plasma membranes from intracellular organelles by centrifugation on self-generating Percoll gradients has been widely used (Pric et al., 1984; Fauvel et al., 1986; Botta et al., 1987; Payrastre et al., 1988). A successful isolation of plasma membrane and sarcoplasmic reticulum from rat myometrium on Percoll gradient is reported here. The entire procedure from the homogenization step to the end can be performed within 2.5 h, whereas other methods using sucrose-gradient centrifugation take 2–14 h just for the sucrose-gradient centrifugation step (Carsten & Miller, 1980; Grover et al., 1980; Soloff & Sweet, 1982). Highly purified plasma membrane and sarcoplasmic reticulum preparations can be obtained and the oxalate-sensitivity of the Ca\(^{2+}\) uptake by the sarcoplasmic reticulum is preserved [for comparison, see Grover & Kwan (1983)].

The characteristics of Ca\(^{2+}\) transport in plasma membrane vesicles prepared in this way are identical to those reported for plasma membranes prepared by sucrose-gradient centrifugation (Enyedi et al., 1988). As with the erythrocyte Ca\(^{2+}\) pump, it has an \(M_{t}\) of 140 000, is responsive to calmodulin, is inhibited by a low concentration of vanadate and is specific for ATP as a substrate (results not shown in detail).

Four independent laboratories have concluded that oxytocin almost completely inhibits the Ca\(^{2+}\)-ATPase, and also in their opinion, the Ca\(^{2+}\) pump activity of
myometrial plasma membranes (Akerman & Wikstrom, 1970; Soloff & Sweet, 1982; Popescu et al., 1985; Huszar, 1986). We have demonstrated that this Ca$$^{2+}$$-ATPase activity is not directly relevant to the Ca$$^{2+}$$ pump (Enyedi et al., 1988). Independently, the inhibition of a constant Mg$$^{2+}$$-ATPase activity by oxytocin in a crude microsomal fraction of rat myometrium has been reported (Missiaen et al., 1988). It has also been pointed out that the inhibition of this Mg$$^{2+}$$-ATPase activity has to be taken into account when the effect of oxytocin on the Ca$$^{2+}$$-ATPase activity is studied. We showed here that this ‘constant’ Mg$$^{2+}$$-ATPase activity is most probably a plasma membrane activity. Based on these findings, it is evident that it is extremely difficult to draw any conclusions from the effects of oxytocin on the Ca$$^{2+}$$-ATPase activity of these membranes.

In this work, we report for the first time the inhibition by oxytocin of the calmodulin-responsive active Ca$$^{2+}$$ transport of subsequently isolated myometrial plasma membrane vesicles, derived from oestrogen (DES)-treated rats. No effect of oxytocin was found when rats were treated with DES plus progesterone, suggesting that oxytocin acts via its extracellular membrane receptors. Oxytocin had its effect by decreasing only the apparent affinity of the calcium pump for Ca$$^{2+}$$ either in the absence or presence of calmodulin; the maximal velocity of the pump was not affected by oxytocin. This provides a sophisticated regulatory mechanism which may be related to the maintenance of elevated cytosolic free Ca$$^{2+}$$. The effect of oxytocin on the calcium transport indicates that this hormone does indeed inhibit the plasma membrane calcium pump as expected (Casteels et al., 1985), but the characteristics of its action are very different from what has been reported, suggesting again that previous observations were, at least in part, on an unrelated activity.

An inhibitory effect of oxytocin on ATP-dependent Ca$$^{2+}$$ ‘binding’ by a sarcoplasmic reticulum preparation has also been reported previously (Carsten, 1974, 1979). In our hands, little or no effect of oxytocin was found on the oxalate-sensitive Ca$$^{2+}$$ transport by the sarcoplasmic reticulum.

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REFERENCES


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