$(Ca^{2+} + Mg^{2+})$-STIMULATED ATPase ACTIVITY OF RABBIT MYOMETRIUM PLASMA MEMBRANE IS BLOCKED BY OXYTOCIN

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1. Introduction

When compared with skeletal muscle, the control of Ca^{2+} movement and hence of contraction/relaxation in smooth muscle is not very well understood (see [1–3]). Sarcoplasmic reticulum, the main regulator of cytosolic (Ca^{2+}) in white skeletal muscle [4], is very poorly developed in smooth muscle, particularly in myometrium [5]. Microsomal fractions isolated from smooth muscle also exhibit very low capacity for ATP-dependent uptake of Ca^{2+} [6–10]. This contrasts greatly to the unusually high capacity and favourable transport kinetics of Ca^{2+} uptake in isolated smooth muscle mitochondria [7,11–13].

Both excitation and contraction of smooth muscle is dependent on the presence of extracellular Ca^{2+} [1], which suggests the possibility of a regulatory role of the plasma membrane. Several cell types, including smooth muscle cells [14–20], have been shown to actively extrude Ca^{2+} from the cytoplasm, across the plasma membrane. The mode of Ca^{2+} extrusion is, perhaps, best characterised in erythrocytes, where Ca^{2+} extrusion is accomplished by a Ca^{2+}-pumping $(Ca^{2+} + Mg^{2+})$-ATPase with quite extraordinary properties [14].

In this communication we describe the identification of a $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity in purified myometrial plasma membrane preparations. The high affinity for Ca^{2+}, and other properties analogous to those of the erythrocyte enzyme, suggest that this enzyme may catalyse ATP-dependent Ca^{2+} extrusion from myometrial smooth muscle cells. The ATPase activity is specifically blocked by physiological concentrations of oxytocin, which supports the proposed function of the enzyme, and suggests a mode of action of the neurohypophyseal peptide hormone in stimulating uterine contractions. A short account of this work has recently appeared [21].

2. Materials and methods

Female rabbits were injected intramuscularly with 1 mg oestradiol/day, for 6–7 days. The rabbits were stunned, and the uteri were quickly removed and placed in ice-cold medium containing 100 mM KCl, 50 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.2. All subsequent procedures were carried out in the cold room. The uterine horns were cut open and the endometrium was gently scraped off. The remaining muscular tissue was cut into small slices (about 5 mm) and homogenised 3 times for 7–10 s using an Ultra-Turrax homogeniser at a tissue/medium ratio of 1:1 (by wt). The suspension was further homogenised with 2 strokes of the pestle of a loosely fitting Potter-Elvehjem glass–Teflon homogeniser, and filtered through 2 layers of cheese-cloth. The filtrate was centrifuged for 60 min at 100 000 × *g* and the pellet was resuspended in 1–2 ml of 0.25 M sucrose, and applied onto the top of a sucrose gradient.

The sucrose gradient was prepared in centrifuge tubes according to the principles in [22,23], but it was discontinuous with layers of 1.47, 1.08, 0.93 and 0.86 M sucrose. Centrifugation followed at 100 000 × *g* for 2.5 h in a swing-out rotor, after which the different fractions were carefully removed with Pasteur pipets. The following fractions were clearly separated. Fraction I (mainly lipid) floated on top of the 0.25 M

sucrose. Fraction II (plasma membranes, PM) settled at the interphase between the 0.25 M and 0.86 M layers. Fraction III + IV (not separated) settled at the interphase between 1.08 M and 1.47 M sucrose. This fraction was further centrifuged at 10 000 × *g* for 10 min. The supernatant (mainly sarcoplasmic reticulum, SR) was designated fraction III, and the pellet (taken up in a small volume of water) was designated fraction IV, containing mainly mitochondria. In addition to these fractions, a pellet settled on the bottom of the sucrose gradient tube. This fraction (whole cells, nuclei) was discarded.

The following marker enzyme activities were assayed by published procedures. 5′-Nucleotidase [24], ouabain-sensitive K^+-stimulated *p*-nitrophenylphosphatase [22], NADH- and NADPH-cytochrome *c* oxidoreductase [25] and cytochrome *c* oxidase [26].

(Ca^{2+} + Mg^{2+})-ATPase activity was assayed in 50 mM Tris–HCl, 20 mM HEPES, pH 7.0, in the presence of 2 mM ATP, 2 mM $MgCl_2$ and different concentrations of Ca^{2+}. The suspensions were preincubated with the biological material for 10 min at 37°C in the absence of ATP, after which the reaction was initiated by ATP addition and continued at 37°C. The reaction was stopped by adding 5% (w/v) ice-cold trichloroacetic acid, followed by centrifugation. Liberated inorganic phosphate was measured from the supernatant according to [27].

(Ca^{2+})-ATPase activity was assayed under identical conditions, except that $MgCl_2$ was omitted. In this case, free (Ca^{2+}) was determined colorimetrically with Arsenazo III [28].

All reaction media and reagents were treated with Chelex to remove Ca^{2+}. The free Ca^{2+} concentration in the media was usually of the order of 1 μM after such treatment as determined with Arsenazo III or a Radiometer F2002 calcium electrode.

Reagents were of highest grade available commercially. Oxytocin (21.5 IU/mg) was kindly donated by OY Medica AB, Helsinki, dissolved in water and lightly buffered with Tris to pH 7.

Protein was determined by the Lowry method [29] using human serum albumin as standard. The protein concentration in the assays was generally ~0.2 mg/ml.

3. Results

Figure 1 shows the profiles of enzyme activity in the fractions II–IV. Panel A, which shows the enzyme activities characteristic for mitochondria (cytochrome

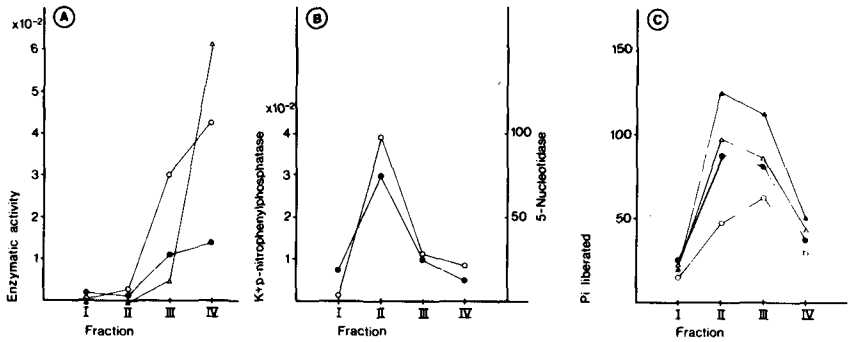

Fig.1. Enzyme distribution in membrane fractions from rabbit myometrium.

Panel A: NADH (○——○) and NADPH (●——●) cytochrome *c* reductase activity expressed as change in extinction at 550 nm/mg protein/min and cytochrome *c* oxidase (△——△) expressed as change in extinction at 550 nm/min/mg protein.

Panel B: K^+-*p*-nitrophenylphosphatase (○——○) activity expressed as ouabain (0.2 mM)-sensitive change of extinction at 400 nm/mg protein/20 min and 5′-nucleotidase (●——●) as nmol inorganic phosphate liberated/mg protein/min.

Panel C: ATPase activity in the presence of 2 mM ATP (○——○) ATP and 1.5 mM $CaCl_2$ (●——●) (free Ca^{2+} ~15 μM), ATP and 2 mM $MgCl_2$ (△——△) or ATP, $MgCl_2$ and 10 μM $CaCl_2$ (▲——▲), expressed as nmol phosphate liberated/mg protein/min.

c oxidase and NADH-cytochrome *c* oxidoreductase) and sarcoplasmic reticulum (NADH- and NADPH-cytochrome *c* reductase), indicates that these activities are virtually absent in fraction II. On the other hand, panel B shows that the traditional markers for plasma membranes, viz, 5′-nucleotidase and ouabain-sensitive K^+-stimulated *p*-nitrophenylphosphatase are concentrated in fraction II, with some contamination into III and IV. We conclude that fraction II is a relatively pure preparation of plasma membranes, whereas III and IV contain mainly SR and mitochondria, respectively, but with some contamination of plasma membranes. This conclusion is also supported by electron microscopy of the different fractions (not shown).

Panel C (fig.1) shows the distribution of various ATPase activities. Phenomenologically, four kinds of ATPase activity may be distinguished, not necessarily implying separate enzymes (see section 4). These are:

(i) A basic ATPase activity apparently requiring neither Ca^{2+} nor Mg^{2+};
(ii) (Ca^{2+})-stimulated ATPase, observed in the absence of added Mg^{2+};
(iii) (Mg^{2+})-stimulated ATPase, observed in the absence of Ca^{2+};
(iv) (Ca^{2+} + Mg^{2+})-stimulated ATPase activity.

Of these, (ii), (iii) and (iv) seem to be enriched in the PM fraction, but are also found in the other fractions.

The (Ca^{2+} + Mg^{2+})-stimulated ATPase activity is very sensitive to the experimental conditions, and is often lost entirely in 1 day upon storage of the PM preparation on ice. This activity is also lost if the homogenisation steps are carried out in dilute sucrose, or if the media contain Ca^{2+} chelators such as EDTA or EGTA. This extreme sensitivity of the (Ca^{2+} + Mg^{2+})-stimulated ATPase activity is similar to that reported for the analogous activity in erythrocytes [17,28]. In erythrocytes, loss of activity appears to be due to dissociation of a Ca^{2+}-binding activator protein from the membrane [30–32]. We have tested a partially purified activator protein from erythrocytes [31] on an inactive myometrial PM preparation, but without success.

Concomitant with the loss of (Ca^{2+} + Mg^{2+})-stimulated ATPase activity on storage there is an increase in the basal (Mg^{2+})-stimulated activity (not shown). This suggests that the latter activity might represent a destruction product of the former, as also proposed earlier for the erythrocyte enzyme [14]. The ATPase activity stimulated by Ca^{2+} alone (Ca^{2+}-ATPase) is not affected by treatments that abolish (Ca^{2+} + Mg^{2+})-stimulated activity. Hence these two activities are likely to represent different enzyme entities (see section 4).

The (Ca^{2+} + Mg^{2+})-stimulated ATPase activity has steep pH and temperature optima at pH 7.0 and 37°C, respectively (not shown, cf. [14,33] for the erythrocyte enzyme).

Figure 2, panel A, shows the time course of ATPase activity of the PM fraction in the presence of Mg^{2+} (filled circles). Addition of 10 μM Ca^{2+} stimulates the activity (open circles), the difference representing the (Ca^{2+} + Mg^{2+})-stimulated ATPase activity. As also shown (open triangles), the latter activity is fully abolished by oxytocin at 4 munits/ml.

Figure 2, panel B, shows ATPase activity in the absence of Mg^{2+}, but in the presence of Ca^{2+} (Ca^{2+}-ATPase). This activity is completely unaffected by oxytocin. The activities stimulated by Mg^{2+} alone and the basal activity without added Mg^{2+} or Ca^{2+} (see fig.1, panel C) are also completely unaffected by the peptide hormone (not shown).

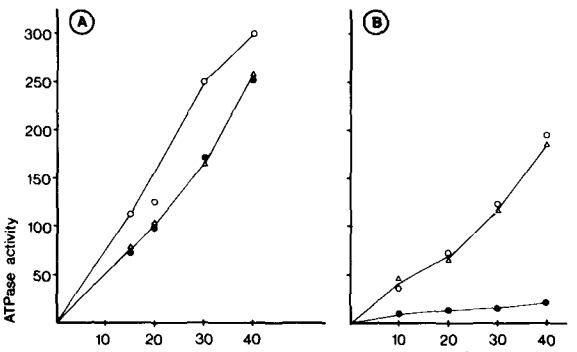

Fig.2. Effect of oxytocin on Ca^{2+}- and (Ca^{2+} + Mg^{2+})-activated ATPase activity in the plasma membrane fraction from rabbit myometrium.

Panel A: Basal activity in the presence of 2 mM $MgCl_2$ (•——•) and 2 mM $MgCl_2$ plus 10 μM $CaCl_2$ (○——○) or in the presence of $MgCl_2$, $CaCl_2$ and 4 munits/ml oxytocin (△——△). Activity expressed as nmol inorganic phosphate liberated/mg protein. Abscissa: time in minutes.

Panel B: Basal ATPase activity (•——•) and in the presence of 1.5 mM $CaCl_2$ (○——○) or $CaCl_2$ and 4 munits/ml oxytocin (△——△). Abscissa: time in minutes.

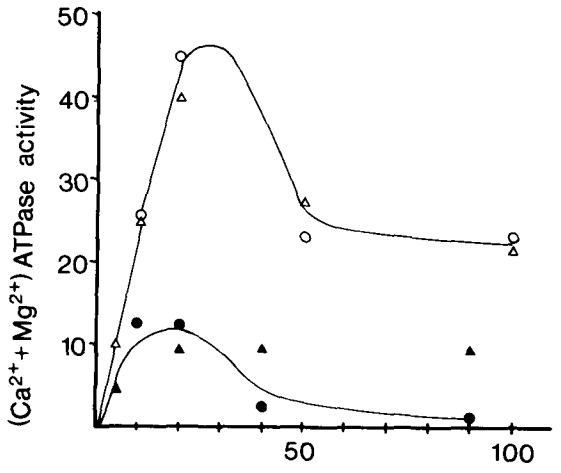

Fig.3. $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity as a function of Ca^{2+} concentration. Ca^{2+}-stimulated activity in the presence of 2 mM $MgCl_2$ and 2 mM ATP (○——○), $MgCl_2$, ATP and 4 units/ml oxytocin (●——●), 20 mM $MgCl_2$ and 2 mM ATP (△——△), or 20 mM $MgCl_2$ ATP and 4 munits/ml oxytocin (▲——▲) expressed as nmol inorganic phosphate liberated/mg protein/min. Abscissa: Ca^{2+} concentration (μM).

Figure 3 shows the $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity of the PM fraction plotted against the concentration of free Ca^{2+}. Both at low and at high Mg^{2+} concentration, the activity is stimulated half-maximally at ~10 μM Ca^{2+}, and appears to fall off at Ca^{2+} concentrations > 30 μM. Again, the inhibitory effect of oxytocin is clearly demonstrated. The inhibitory effect of oxytocin has been reproducible in more than a dozen of experiments with different oestrogen-treated rabbits. In a few instances the specific $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity was destroyed during isolation.

The half-maximal inhibitory effect of oxytocin on $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity varies somewhat between different preparations, but is $\leqslant$ 0.4 mU/ml (~10^{-9} M) at 0.2 mg protein/ml, corresponding to ~5×10^{-12} mol oxytocin/mg protein.

The inhibitory effect of oxytocin is absent or much less prominent when tested upon the $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity of the SR fraction (fraction III, not shown). A slight inhibition may well be attributed to occasional contamination of the SR fraction with plasma membranes (cf. fig.1B). Preliminary runs of the PM fraction in SDS–polyacrylamide gel electrophoresis suggest that only 2–3 major polypeptides are present, supporting the relative purity of this preparation.

4. Discussion

Our results suggest that the plasma membrane of the myometrium cells contains a $(Ca^{2+} + Mg^{2+})$-stimulated ATPase, which in its properties greatly resembles an analogous enzyme in the erythrocyte membrane [14,17]. The ATPase activity stimulated by Ca^{2+} alone (Ca^{2+}-ATPase) is presumably a separate molecular entity, as it is in erythrocytes, where it has been shown to be unrelated to Ca^{2+} transport [14]. The myometrial activity requires > 0.1 mM Ca^{2+} for half-maximal stimulation in contrast to the high Ca^{2+} affinity of the $(Ca^{2+} + Mg^{2+})$-stimulated ATPase activity. The $(Ca^{2+} + Mg^{2+})$-stimulated activity may well represent an outward-directed Ca^{2+}-transport system, energised by ATP hydrolysis, as is also the case in erythrocytes. This idea is further supported by our finding that this activity is stimulated by low concentrations of calcium, comparable to those prevailing intracellularly. It may be added that if Ca^{2+} influx into the cytoplasm is needed concomitant with excitation/concentration coupling in smooth muscle, as may be inferred from the dependence of contractions of extracellular Ca^{2+}, the muscle cell must have some means of actively extruding Ca^{2+} from the cell. Furthermore, Ca^{2+} transport has been demonstrated in myometrial plasma membrane preparations [19]. Evidence has also been presented [34] against the possibility of calcium extrusion from the myometrium cell in exchange for Na^+. We have as yet been unable to detect any Ca^{2+} transport in our preparations, which may, however, contain mainly open fragments of the membrane.

Ca^{2+} flux studies in uterine smooth muscle have so far failed to demonstrate active extrusion of the cation [34–36]. However, due to the large interstitial space of the tissue, it seems quite possible that a relatively small flux across the plasma membrane would go easily unnoticed. In preliminary experiments, we have in fact recently observed a linear component of Ca^{2+} efflux from uterine slices, which is dependent on energy metabolism and is inhibited by oxytocin. This finding therefore supports the notion that oxytocin blocks Ca^{2+} extrusion by inhibiting the $(Ca^{2+} + Mg^{2+})$-dependent ATPase. In the light of these

findings, it may not be unreasonable to suggest that this effect of oxytocin may, at least in part, represent the basis of the physiological action of this hormone on the uterus. As demonstrated [37], myometrial membrane preparations bind oxytocin specifically with very high affinity. The specific oxytocin-binding site has more recently been localised to the myometrial plasma membrane [38]. Since the binding affinity is quite comparable with the oxytocin concentrations required to inhibit $(Ca^{2+} + Mg^{2+})$-stimulated ATPase, and with those required to stimulate uterine contractions in vivo, it is possible that the inhibition of Ca^{2+} efflux from the myometrial cell is the main physiological effect of oxytocin.

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