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The effect of Ca deprivation and of Ca-blocking drugs on oxytocin-induced contractions of the male mouse anococcygeus

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Abstract—Oxytocin (4 nM)-induced contractions of the male mouse anococcygeus were rapidly and completely lost in EGTA (2 mM)-containing, Ca-free Krebs solution. Contractions were also lost, although more slowly, in Ca-free Krebs solution without EGTA; under such conditions, readdition of Ca did not by itself cause contraction, but readdition of Ca (0·1–2·5 mM) in the presence of 4 nM oxytocin resulted in a rapid contractile response. These Ca-induced responses, in the presence of oxytocin, and those to oxytocin in normal Ca-containing Krebs solution, were unaffected by nitrendipine (0·01–1 μM). Contractions to oxytocin were completely blocked by the calmodulin antagonists trifluoperazine (50 μM) and W-7 (75 μM). It is concluded that oxytocin-induced contraction of the mouse anococcygeus does not require opening of nitrendipine-sensitive Ca channels, and there is no Ca-independent component of the contractile response; the cellular mechanisms linked to the oxytocin receptor in the anococcygeus are therefore different from those in the uterus.

Anococcygeus muscles, isolated from male mice, contract in the presence of oxytocin (Gibson et al 1984), the sensitivity of the tissue being similar to that of established oxytocin bioassay preparations (Botting & Gibson 1985). Experiments with selective agonists and antagonists have shown that the oxytocin receptors in the anococcygeus closely resemble those found in the uterus (Gibson 1986). Therefore, the mouse anococcygeus provides a useful model with which to broaden investigations into the mode of action of the polypeptide, since the majority of such studies have so far been carried out on uterine tissue. In particular, there has been increasing interest in the role of calcium ions (Ca) in oxytocin-induced contractions of the uterus, and it has been suggested that such contractions result from mobilization of extracellular Ca through voltage-operated channels, which are sensitive to Ca channel blocking drugs, and from the release of Ca from intracellular stores (Barnes & Senior 1985; Batra 1986; Edwards et al 1986). However, the uterus continues to contract to oxytocin, for up to 50 h, in the absence of Ca and in the presence of the calmodulin antagonist, trifluoperazine (Sakai et al 1981; Ashoori et al 1985); these observations had led to the proposal that a component of the uterine contraction to oxytocin involves a Ca-independent process (Ashoori et al 1985). It was therefore of interest to investigate whether the same mechanisms operate in the mouse anococcygeus, and thereby to determine whether the cellular mechanisms activated by oxytocin are receptor-specific or organ-specific. Thus, the present study sought to answer the following questions: 1) are oxytocin-induced contractions of the mouse anococcygeus inhibited by the Ca channel blocking drug nitrendipine, and 2) is there any evidence for a Ca-independent component of the contractile response to oxytocin?

Methods

Male mice (LACA strain; 25–35 g) were killed by stunning and exsanguination. The two anococcygeus muscles from each animal were dissected as described previously (Gibson & Wedmore 1981), and were set up in series, joined at the ventral bar, in 1 mL glass organ baths. The bathing medium was Krebs bicarbonate buffer, the 'normal' composition of which was: (mM) NaCl 118·1; KCl 4·7; $MgSO_4$ 1·0; $KH2PO_4$ 1·2; $NaHCO_3$ 25·0; $CaCl_2$ 2·5; glucose 11·1. The medium was maintained at 37°C and was gassed continuously with 95% O_2: 5% CO_2. A resting tension of 200–400 mg was placed on the tissue and changes in tension recorded with a Grass FTO3 force-displacement transducer attached to either a Lectromed or Graphtec pen recorder. Muscles were allowed to equilibrate for 45 min before beginning the experiment.

Oxytocin was added to the organ bath in volumes not exceeding 50 μL, and was in contact with the tissues for 4 min, or until any consequent rise in tone had reached a peak. After washout, the muscle was allowed to rest for 20 min before a further concentration of oxytocin was added.

Ca-free Krebs solution was prepared by simple omission of $CaCl_2$; no compensation was made for the resultant small change in tonicity or Cl concentration. In experiments involving Ca readdition, the Ca-free Krebs solution also contained 1 μM phentolamine to ensure that any contractions observed did not result from release of noradrenaline from the sympathetic nerve endings within the tissue (Gibson & Wedmore 1981).

Drugs used were: ethylene glycol-bis-(β-amino ethylether)-*N,N,N',N'*-tetra-acetic acid (EGTA, Sigma), nitrendipine (donated by Dr J. H. Botting, stock solution of 1 mM dissolved in absolute ethanol and diluted thereafter with 0·9% saline), oxytocin (Syntocinon, Sandoz), phentolamine mesylate (Ciba), trifluoperazine hydrochloride (Sigma), W-7 (*N*-6-aminohexyl-5-chloro-1-naphthalene sulphonamide, Sigma).

Statistical analysis was by Student's *t*-test.

Results

As found previously (Botting & Gibson 1985), oxytocin (0·1–10 nM) produced concentration-related contractions of the mouse anococcygeus muscle; 4 nM produced a suitable submaximal response (80 ± 3% of the maximum, mean ± s.e., n = 8) and was used in all subsequent experiments.

Replacement of normal Krebs solution with Ca-free solution containing 2 mM EGTA resulted in complete loss of the oxytocin-induced contraction (Fig. 1a); in a total of 6 such experiments there was no evidence of any residual response to oxytocin, even on first exposure to the peptide after Ca removal. Replacement of normal Krebs solution with Ca-free solution lacking EGTA caused a more gradual decline in the contractile response to oxytocin (Fig. 1b), but even here responses were totally abolished after 90 min. In such Ca-free medium (without EGTA), when responses to oxytocin had disappeared, readdition of Ca (0·1–2·5 mM) did not by itself cause muscle contraction. However, readdition of Ca in the presence of 4 nM oxytocin (added 2 min earlier) resulted in a rapid contractile response (Fig. 2a); these Ca-induced, oxytocin-dependent contractions were concentration-related and were unaffected by the Ca channel blocking drug nitrendipine (Figs. 2a, b). Contractile

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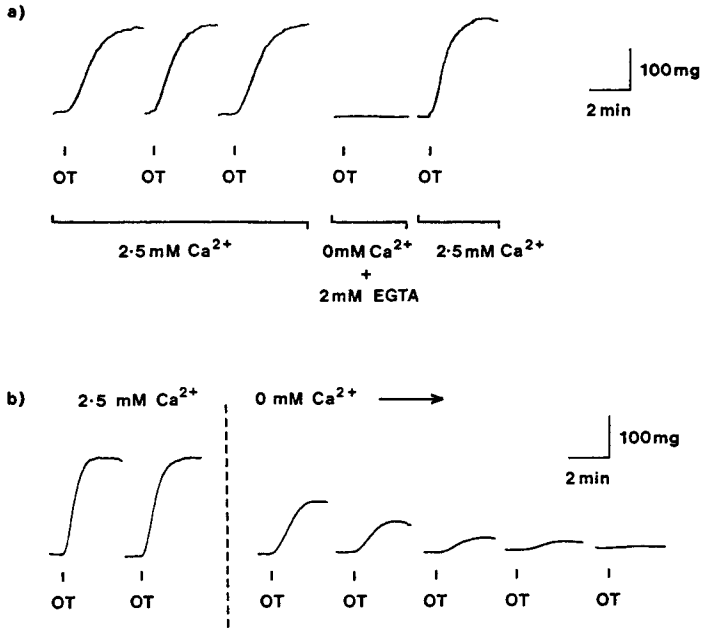

FIG. 1. (a) Contractions of a mouse anococcygeus muscle to 4 nM oxytocin (OT) and the effect on these of changing from normal Krebs solution (containing 2·5 mM Ca) to Ca-free solution containing 2 mM EGTA. Each break in the trace represents a time interval of 20 min. Ca deprivation resulted in a complete, but reversible, loss of response to OT. (b) Contractions of a mouse anococcygeus muscle to 4 nM oxytocin (OT) and the effect on these of changing from normal Krebs solution (containing 2·5 mM Ca) to Ca-free solution without EGTA. Each break in the trace represents a time interval of 20 min. In Ca-free medium, there was a gradual, and eventual complete, loss of response to OT.

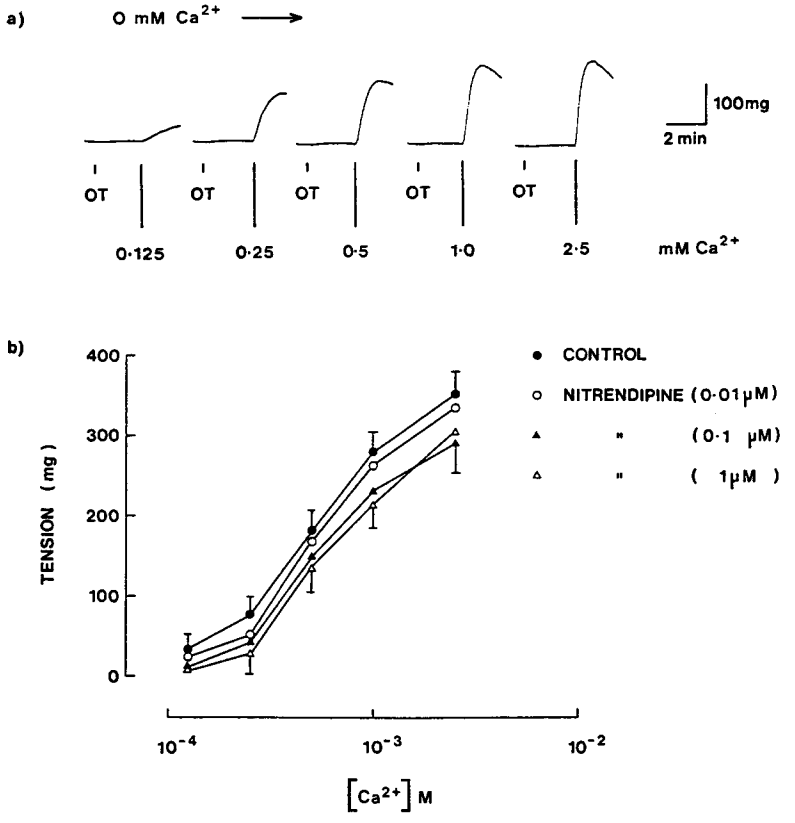

FIG. 2. (a) Contractions of a mouse anococcygeus muscle to increasing concentrations of Ca in the presence of 4 nM oxytocin (OT) (added 2 min earlier); the muscle had been immersed in Ca-free Krebs solution (without EGTA) for sufficient time to allow complete loss of response to OT alone (as in 1b). Each break in the trace represents a time interval of 20 min. (b) Concentration-response curves for Ca-induced OT-dependent contractions of mouse anococcygeus muscle in Ca-free medium without EGTA (as in a), and the effect on these curves of nitrendipine. Each point is the mean ± s.e. from at least 6 individual muscle preparations. Nitrendipine had no significant effect on these contractions.

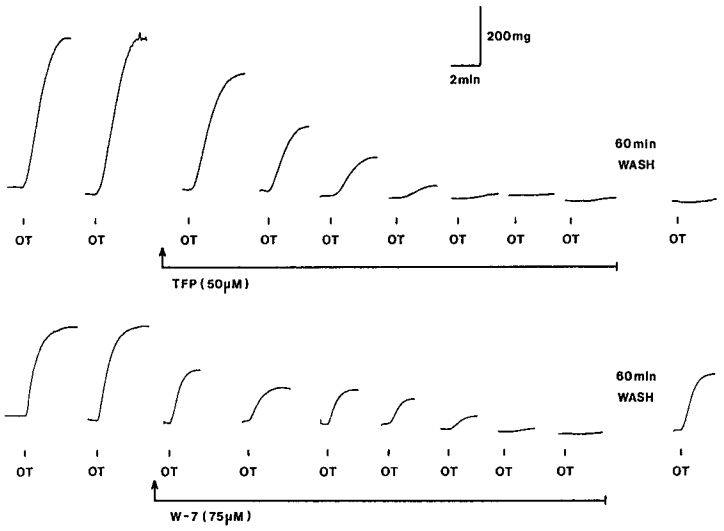

FIG. 3. Contractions of mouse anococcygeus muscles to 4 nM oxytocin (OT) and the effect on these of trifluoperazine (TFP) and W-7. Each break in the trace represents a time interval of 20 min. Both calmodulin antagonists produced a progressive decline, and eventual loss, of responses to OT. The effect of TFP was not reversed 60 min after washout, but that of W-7 showed partial reversal.

responses to 4 nM oxytocin (340 ± 26 mg tension, $n = 8$) in normal Krebs solution were also unaffected in the presence of 200 nM nitrendipine (318 ± 42 mg tension, $n = 6$, $P > 0.05$).

The calmodulin antagonists trifluoperazine (50 μM; Levin & Weiss 1979) and W-7 (75 μM; Tanaka et al 1982) caused a progressive decline in the response to oxytocin (Fig. 3). In both cases, contractions were totally abolished after 120 min. The inhibitory effect of W-7 was partially reversed 60 min after washout, but that of trifluoperazine was unchanged (Fig. 3).

Discussion

Oxytocin-induced contractions of the rat uterus are remarkably resistant to Ca deprivation (Sakai et al 1981) and persist for up to 50 h when the tissue is bathed in Ca-free medium (Ashoori et al 1985). Further, the calmodulin antagonist trifluoperazine has little effect on such contractions (Ashoori et al 1985). One explanation put forward to explain these results is that a Ca-independent mechanism may be involved in the uterine response to oxytocin (Ashoori et al 1985). Several observations of the present study clearly show that a similar mechanism does not operate in the mouse anococcygeus. First, oxytocin-induced contractions of the anococcygeus were rapidly and completely lost on changing to Ca-free medium containing EGTA. Secondly, under less severe conditions of Ca deprivation (Ca-free Krebs without EGTA), responses to oxytocin declined more slowly but, even here, they were totally abolished after 90 min. Finally, the calmodulin antagonists trifluoperazine (Levin & Weiss 1979) and W-7 (Tanaka et al 1982) both produced complete inhibition of responses to oxytocin.

When responses to oxytocin had been lost in Ca-free medium (without EGTA), readdition of Ca in the presence of oxytocin resulted in rapid contraction. Since readdition of Ca in its absence did not cause contraction it seems that oxytocin allows access of extracellular Ca into the cell. If so, the Ca does not gain access through channels sensitive to block by dihydropyridine Ca channel blocking drugs, since nitrendipine did not block Ca-induced, oxytocin-dependent responses or contractions induced by oxytocin in normal Krebs solution. Thus, unlike the uterus (Barnes & Senior 1985; Edwards et al 1986), voltage-operated channels of the L-type (Bolton 1979; Bolton et al 1988) are not involved in contractions of the anococcygeus to oxytocin. Some preliminary experiments in our laboratory suggest that dihydropyridine-sensitive Ca channels do exist in the mouse anococcygeus since contractions to high potassium are blocked by nitrendipine (0·1–500 nM); however, contractile responses of the anococcygeus to potassium are complex (Gibson & James 1977) and more detailed studies are necessary to characterize the various Ca channels present in the tissue. The observation that responses to oxytocin were rapidly lost in EGTA-containing, Ca-free Krebs solution, even on first exposure to the peptide after changing to such medium, supports the suggestion that extracellular Ca is involved in the contractile response of the anococcygeus to oxytocin (van Breemen et al 1982; Bohr 1988).

In conclusion, the questions posed in the introduction can now be answered: oxytocin-induced contractions of the mouse anococcygeus are not blocked by nitrendipine, and there is no evidence for involvement of a Ca-independent process in the contractile response. In these respects, the mechanisms activated by oxytocin in the anococcygeus differ from those in the uterus and therefore the cellular systems linked to the oxytocin receptor are not uniform, but vary according to the tissue in which they are located.

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Bioavailability of intramuscular vitamin E acetate in rabbits

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Abstract—The bioavailability of α-tocopherol acetate and α-tocopherol (vitamin E) was assessed in male rabbits given 50 mg kg^{-1} doses according to a randomized design. After intramuscular injection of α-tocopherol acetate in colloidal aqueous solution, a mean absolute bioavailability of 65% was calculated for the acetate and 35% for the physiologically active compound, α-tocopherol. Comparison of the kinetic profiles after intravenous and intramuscular administration of the acetate and intravenous injection of α-tocopherol, revealed absorption of α-tocopherol acetate from the site of injection and hydrolysis of the acetate to be potential limiting steps in the bioavailability of α-tocopherol. Intramuscularly injected α-tocopherol acetate in olive oil (the only formulation available in a few European countries) proved completely bio-unavailable. It thus appears necessary to re-assess the utility of current vitamin E supplementation, since the only formulations available offer poor bioavailability.

α-Tocopherol (vitamin E), one of the biological antioxidants, is currently receiving attention concerning its efficacy in preventing or reducing the incidence of severe clinical conditions associated with adverse oxidation events in premature newborns (retrolental fibroplasia, intraventricular haemorrhage, bronchopulmonary dysplasia, haemolytic anaemia) and in adults (coronary, rheumatic and hypertensive heart diseases) (Bieri et al 1983; Roberts & Knight 1987).

α-Tocopherol is practically insoluble in water and is oxidized by atmospheric oxygen and light (Sebrell & Harris 1954). Because of their greater stability, the esters of α-tocopherol (acetate, succinate, nicotinate) are commonly used in different formulations, particularly intramuscular preparations of the acetate. Oral administrations are bioavailable but have a slow absorption rate (particularly in the preterm newborn, who have immature gastrointestinal function and in whom pathological states may affect oral absorption (Morselli et al 1980). Thus is it of limited use in acutely raising blood levels (Bell et al 1979). Then, too, intravenous administration resulted in a tragedy (Martone et al 1986), with the deaths of 38 infants who had received the drug. However, the esters, also, are poorly soluble in aqueous solutions. So, different formulae (micellar aqueous dispersion, colloidal aqueous solutions, olive and sesame oil solutions) have been prepared to improve the bioavailabilty of α-tocopherol acetate and also its hydrolysis to α-tocopherol, the active moiety.

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There have been a number of studies about the bioavailability of vitamin E in animals (Newmark et al 1975; Phelps 1981), human neonates (Bougle et al 1986) and adults (Bateman & Uccellini 1985; Baker et al 1986). These studies, using different doses, timing, routes of administration and formulations, tend to give piecemeal information rather than a systematic picture. Thus, to date, no information is available concerning the intramuscular bioavailability of the one parenteral formulation in olive oil available in Italy and other European countries.

The present study was designed to compare the disposition profile of this formulation and a standard colloidal aqueous solution, currently available only in a few countries.

Materials and methods

According to a randomized design six male New Zealand rabbits (Charles River, Italy), 3–3·5 kg, were given 50 mg kg^{-1} of: i) (±)-α-tocopherol acetate in colloidal solution (Ephynal, Hoffman-LaRoche, Switzerland) intravenously (i.v.) (in one of the marginal ear veins) and intramuscularly (i.m.) (deep into the thigh muscle); ii) (±)-α-tocopherol i.v., as a galenical aqueous dispersion (Phelps 1981); iii) (±)-α-tocopherol acetate in olive oil (Evion Forte, Bracco, Italy).

Blood samples (0·5–1 mL) were drawn from a vein in the non-injected ear in heparinized syringes at various times up to 72 h. The samples were stored at −20°C until assay. Blood concentrations of α-tocopherol acetate and α-tocopherol were determined by the HPLC method of Celardo et al (1988).

A two-week wash-out period was left between one treatment and the next, to allow a return to similar blood trough levels of vitamin E in all the rabbits after each treatment (4·3 ± 0·8). Hematocrit, body weight, food and water consumption were checked daily during the study.

Blood concentration-time data after different doses, minus the basal value, were analysed according to a non-compartmental analysis and the parameters were obtained from the usual relationships (Gibaldi & Perrier 1982). The highest sample concentration observed was taken as the peak concentration. The "lag time (observed)" for absorption or formation was estimated as the midpoint between the times of collection of the first sample above assay sensitivity (for α-tocopherol acetate) or trough level (for α-tocopherol) and the sample immediately before it. Total body clearance (CL) after i.v. administration was calculated as $CL = dose^{iv}/AUC^{iv}$, where AUC is the area under the curve from zero to infinity, estimated using the trapezoidal