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THE ACTION OF 2-O-METHYLTYROSINE-OXYTOCIN ON THE RAT AND RABBIT UTERUS: EFFECT OF SOME EXPERIMENTAL CONDITIONS ON CHANGE FROM AGONISM TO ANTAGONISM

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2-O-Methyltyrosine-oxytocin (referred to briefly as methyloxytocin in this paper) is an analogue of oxytocin formally derived from the native hormone by methylation of the phenolic hydroxyl group in the tyrosine side chain. This analogue, synthesized independently by Law & du Vigneaud (1960) and by Jošt, Rudinger & Šorm (1961, 1963), was found to inhibit the pressor action of vasopressin in the anaesthetized rat (Law & du Vigneaud, 1960) and the uterotonic effect of oxytocin on the isolated rat uterus (Beránková, Rychlík, Jošt, Rudinger & Šorm, 1961). The inhibition was highly specific, reversible, and apparently competitive (Rudinger & Krejčí, 1962; Bisset, 1962). However, in some experiments methyloxytocin exhibited oxytocin-like uterotonic action; further investigation (Krejčí, Poláček, Kupková & Rudinger, 1964) showed that the inhibitory properties of the analogue appear only under certain experimental conditions, defined both by extrinsic factors (ionic composition and temperature of the organ bath) and by the condition of the organ preparation (hormonal state). This paper presents a more detailed account of the action of methyloxytocin on the isolated rat uterus under various conditions and describes its action on the isolated rabbit uterus and the rat uterus *in vivo*; a preliminary report of the results has been given (Krejčí, Poláček & Rudinger, 1966).

METHODS

Isolated rat uterus

The rats were virgin females of the Wistar strain (farm Konárovice), weight 200–250 g. According to the hormonal state or pretreatment they constituted two groups:

(1) Oestrogen-dominated animals were either (a) normal rats treated with a single dose (10–20 μg) of oestradiol dipropionate in oil intramuscularly 24–48 hr before the experiment, or (b) spayed rats one week after ovariectomy given twice 10–20 μg of the oestrogen 48 and 24 hr before the experiment, or (c) normal rats given twice 50 μg of the oestrogen on successive days.

(2) Rats whose vaginal smears showed a preponderance of leucocytes. These were (a) spayed rats given 10 μg oestradiol propionate daily for 3 days, then checked daily by vaginal smears until leucocytes appeared (generally 4–7 days after the last dose of oestrogen), and used within 21 days of

this point; (b) spayed, oestrogenized rats given 2–3 mg progesterone daily in oil intramuscularly for 3–8 days after cessation of oestrogen treatment and (c) pseudopregnant rats (Rothchild & Schubert, 1963) which on the day of natural oestrus received 10 mg progesterone and were used 2–6 days later (control by vaginal smears).

The uterine horns were cut open longitudinally and the middle sections, corresponding as nearly as possible to a length of 1 cm in the condition of the organ *in situ*, were promptly suspended in the organ bath. The strips were loaded with 1.5 g under isometric recording conditions and then allowed to relax for 1 hr. The organ bath was filled with 15 ml. of the appropriate medium and a current of 95% oxygen–5% carbon dioxide mixture, or of air, was passed through; the pH was checked at intervals. Holton's (1948) medium (NaCl, 154; KCl, 5.4; $NaHCO_3$, 6; glucose, 2.77 mM) and Munsick's (1960) medium (NaCl, 114; KCl, 6.2; $NaHCO_3$, 30; NaH_2PO_4, 1; glucose, 2.77 mM) were used, $CaCl_2$ and $MgCl_2$ being added to the concentration stated for individual experiments from stock solutions standardized by chelatometric titration. Unless otherwise stated, the temperature of the organ bath was 30° C.

The effect of increased potassium ion concentration was studied at 25° C (see Daniel, Sehdev & Robinson, 1962) using the K_2SO_4-Ringer medium of Edman & Schild (1962) (K_2SO_4, 126; KCl, 5.6; $KHCO_3$, 3.6; glucose, 5.55 mM) and Krebs medium (NaCl, 133.4; KCl, 4.74; KH_2PO_4, 1.18; $NaHCO_3$, 9.94; glucose, 5.55 mM) with the concentration of KCl increased to 47, 70, or 100 mM and a corresponding reduction in the concentration of NaCl (Marshall, 1964). The amount of $CaCl_2$ added is given for the individual experiments.

Contractions were measured under approximately isometric conditions using a flat spring tension recorder with a change in length of the muscle strip by not more than 5% of its length after relaxation, and were displayed on a smoked drum.

Dose–response relations were determined using logarithmically increasing doses, with rinsing between successive doses, or by a cumulative dose procedure (Ariëns & de Groot, 1954), the concentration of the compound tested in the organ bath being doubled, without rinsing, at 45–60 sec intervals until the maximal response was reached. Cumulative dose curves were used in the calculation of the pD_2 (the negative logarithm of the dose evoking one-half the maximal response).

In studies of inhibition by methyloxytocin, the analogue was added to the organ bath generally 3 min before the oxytocin. Oxytocin was tested in 2–3 doses in varying sequence in the presence and absence of the inhibitor, or, in cumulative dose experiments, alternately in the presence and absence of the inhibitor.

Isolated rabbit uterus

Adult virgin female rabbits were used without pretreatment, or after injection with 100–300 μg oestradiol dipropionate 48 hr before the experiment. Strips about 20×5 mm were cut from the centre portions of the uterine horns and set up as described for the rat uterus above.

Rat uterus in situ

Oestrogenized rats under ethanol narcosis were used. After laparotomy one uterine horn was incised in its cervical part; a thin polyethylene cannula was introduced into the uterine cavity and secured by a ligature; the uterus was filled with physiological saline at an excess pressure of 10–20 mm water and the pressure changes were recorded on a smoked drum using a float-type manometer (Řežábek & Souček, 1962). The animal was kept in an incubator at 37° C. Samples were injected through a cannula into the femoral vein. The spontaneous activity of the uterus during 5 min preceding a test dose was calculated as the product of the pressure and frequency (in analogy to the Montevideo units of Caldeyro-Barcia, Sica-Blanco, Poseiro, Gonzalez-Panizza, Méndez-Bauer, Fielitz, Alvarez, Pose & Hendricks (1957), except that the pressure was expressed arbitrarily in mm of the kymographic record). The activity after injection of the sample was calculated in similar units for 5-min intervals until it returned to the "spontaneous" value. The uterotonic effect was equated to the difference between the stimulated activity over the whole duration of increased activity and the spontaneous activity for a corresponding period.

Materials

The Czechoslovak National Standard of Posterior Pituitary Extract, assayed against the Third International Standard of Oxytocic, Vasopressor, and Antidiuretic Substances (Bangham & Musset, 1958), served for reference in quantitative assays. The effect of methyloxytocin on the uterotonic effect of oxytocin was studied using synthetic oxytocin (SPOFA). Samples of methyloxytocin purified by countercurrent distribution and stored as the lyophilized powder at −8° C, or in ampoules as a sterile dilute solution (0.1 mg/ml., pH 3.5) at 0° C, were used.

RESULTS

Types of response of the isolated rat and rabbit uterus to methyloxytocin

Rat or rabbit uterine strips showing spontaneous contractions (whatever the experimental conditions) responded to methyloxytocin, as they did to oxytocin, by an increase in the intensity and frequency of the contractions and (at higher doses) by an increase in tone.

The response to methyloxytocin of uterine strips not undergoing spontaneous contractions varied qualitatively in dependence on the hormonal state of the organ, the calcium concentration in the medium, and the temperature of the organ bath. These variations affected the frequency of the contractions, the dose–response relations and maximal response, and the effect of the analogue on the response to oxytocin. To relate the behaviour of methyloxytocin to the experimental conditions (Tables 1 and 2) the responses may be classified in three types, corresponding loosely to behaviour of the analogue as a full agonist (A), partial agonist (B), and antagonist (C) of oxytocin.

TABLE 1

RESPONSE OF RAT AND RABBIT UTERINE STRIPS TO METHYLOXYTOCIN IN DEPENDENCE ON THE EXPERIMENTAL CONDITIONS

Medium*	Calcium mM	Spontaneous contractions	Responses of type† A	B	C
		Oestrogen-treated rats (groups 1a and 1b)‡			
Holton	0·6	2/13	6/13	3/13	2/13
Holton	0·3	3/25	4/25	5/25	13/25
Munsick	0·6	2/20	2/20	4/20	12/20
Munsick §	0·6	0	0	3/10	7/10
		Rats after cessation of oestrogen treatment (group 2a)‡			
Holton	0·6	7/14**	0	4/14	3/14
Holton	0·3	6/13**	0	5/13	2/13
		Rats pretreated with progesterone (group 2b)‡			
Holton	0·6	4/5	0	1/5	0
Holton	0·3	3/5	0	2/5	0
Munsick	0·6	4/4	0	0	0
		Pseudopregnant rats (group 2c)‡			
Holton	0·6	4/5	1/5	0	0
Holton	0·3	2/5	1/5	2/5	0
		Untreated rabbits			
Holton	1·2	1/8	0	1/8	6/8
Holton	0·6	1/8	0	0	7/8

* At 30° C unless otherwise stated. † For definition see results section, p. 509–510. ‡ For details. see Methods section, p. 505–507. § At 20° C. ** Spontaneous contractions of low amplitude and frequency, only slightly increased by 7 or 25 m-u/ml. oxytocin.

TABLE 2

RELATION BETWEEN SENSITIVITY TO OXYTOCIN AND RESPONSE TO METHYLOXYTOCIN
Uterine strips from oestrogenized rabbits in Holton's medium with 0·6 mM Ca^{2+} at 30° C

Minimum active dose of oxytocin, m-u/15 ml.	Proportion of inhibitor responses
<2	2/9
10–20	15/20
>60	8/8

Type A. Methyloxytocin exerted a uterotonic effect qualitatively similar to that of oxytocin in the intensity and frequency of the contractions. Log dose–response curves for the analogue obtained with washing out between doses and by the cumulative dose procedure were indistinguishable, and parallel to those for oxytocin.

Type B. In comparison with a dose of oxytocin eliciting a first contraction of approximately the same intensity, methyloxytocin caused rhythmic contractions of lower frequency and, generally, rapidly decreasing tension; sometimes the response was confined to a single contraction (Fig. 1). Log dose–response curves recorded by the cumulative dose procedure had lower slopes, and lower maxima, than those determined with washing out between doses: the latter remained roughly parallel to the log dose–response curves for oxytocin (determined by either procedure) (Fig. 2).

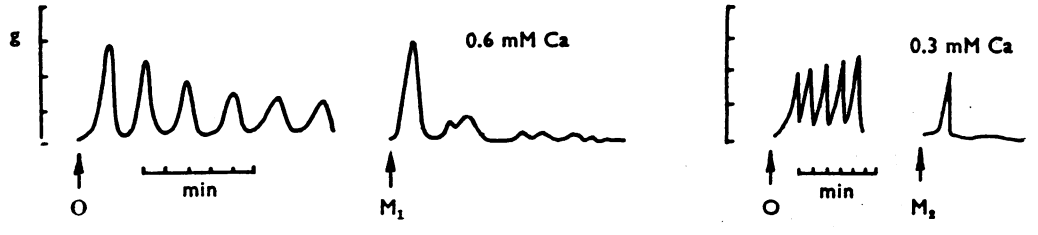

Fig. 1. Action of oxytocin and methyloxytocin on the oestrogenized rat uterus. Oestrogenized rat uterus (Methods section group 1a), Holton's medium with Ca concentration as shown, 30° C; O oxytocin (5 m-u), M_1 methyloxytocin (2.5 μg), M_2 methyloxytocin (5 μg). Note rapid damping of response to methyloxytocin.

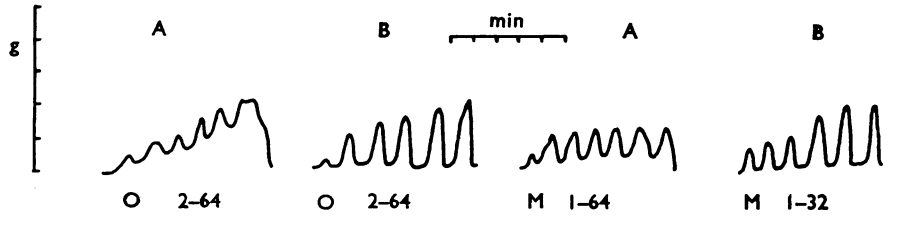

Fig. 2. Response of oestrogenized rat uterus to oxytocin and methyloxytocin in single and in cumulative doses. Oestrogenized spayed rat (Methods section group 1b), Holton's medium with 0.3 mM Ca, 30° C; O oxytocin in m-u, M methyloxytocin in μg. A: cumulative dose experiments. B: experiments with rinsing between individual doses.

Type C. Methyloxytocin showed no uterotonic effect, or very low uterotonic activity without regular dependence on dose (Fig. 3). The action of added oxytocin was partly or wholly inhibited, depending on the dose. In the presence of the analogue the response to oxytocin may show not only decreased intensity, but decreased frequency, or both. In some cases there was only a single contraction in response to oxytocin (Fig. 4).

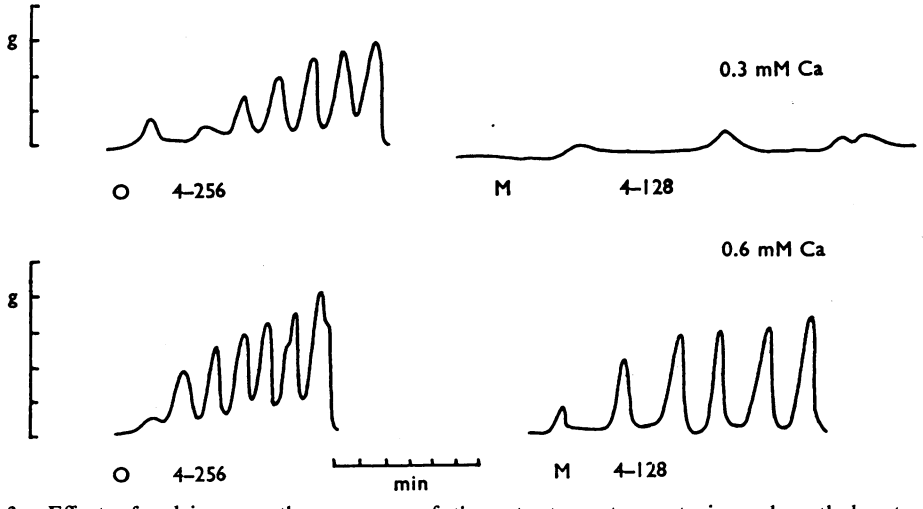

Fig. 3. Effect of calcium on the response of the rat uterus to oxytocin and methyloxytocin in cumulative dose experiments. Ca concentrations as shown, other conditions and symbols as for Fig. 2.

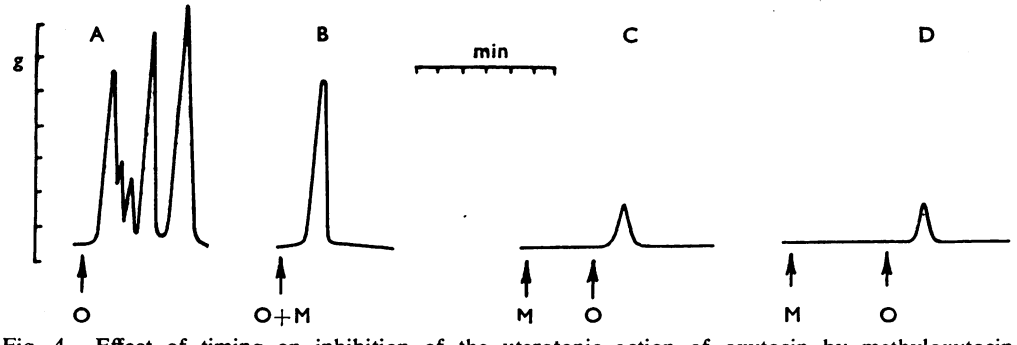

Fig. 4. Effect of timing on inhibition of the uterotonic action of oxytocin by methyloxytocin. Oestrogenized rabbit uterus *in vitro*, Holton's medium with 0.6 mM Ca, O oxytocin (10 m-u), M methyloxytocin (1 μg). Note change from rhythmic activity in absence of inhibitor (A) to single contractions (B—D).

Uterotonic potency of methyloxytocin in vitro

In 14 Schild-type assays carried out on rat uterine strips in Holton's medium under conditions where the responses to methyloxytocin and oxytocin were qualitatively identical (Type A above) the potency of methyloxytocin was found to be 1.0 ± 0.2 u/mg in the presence of 0.3 mM Ca^{2+} and 1.5 ± 0.5 u/mg in 0.6 mM Ca^{2+}, that is, about 0.2% of the potency of oxytocin.

Effect of the hormonal state

The types of response observed with uterine strips from rats under different hormonal regimes are summarized in Table 1. In experiments with rabbit uteri the incidence of inhibition was generally higher than with rat uteri; on organs taken from untreated rabbits (threshold dose for oxytocin 20–25 m-u/15 ml.) methyloxytocin almost invariably acted as an inhibitor (Table 1) whereas on organs from oestrogenized rabbits it had oxytocin-like action in a fair proportion of the experiments, apparently in dependence on the sensitivity of the preparation (Table 2).

Effects of calcium and magnesium ions

The results in Table 1 suggest that the response of the rat uterus to methyloxytocin depends on the calcium concentration (see also Fig. 3). The effect of the calcium concentration in Holton's medium (without magnesium) on the response of uteri from oestrogenized rats to oxytocin and to methyloxytocin was studied in additional experiments using the cumulative dose procedure. The maximal tension achieved at high doses in each experiment (expressed as a percentage of the maximal response to oxytocin in 0.3 mM calcium) and the pD_2 values are plotted as functions of the calcium concentration in Fig. 5. The effect of added magnesium in the presence of 0.3 mM calcium is similarly shown in Fig. 6.

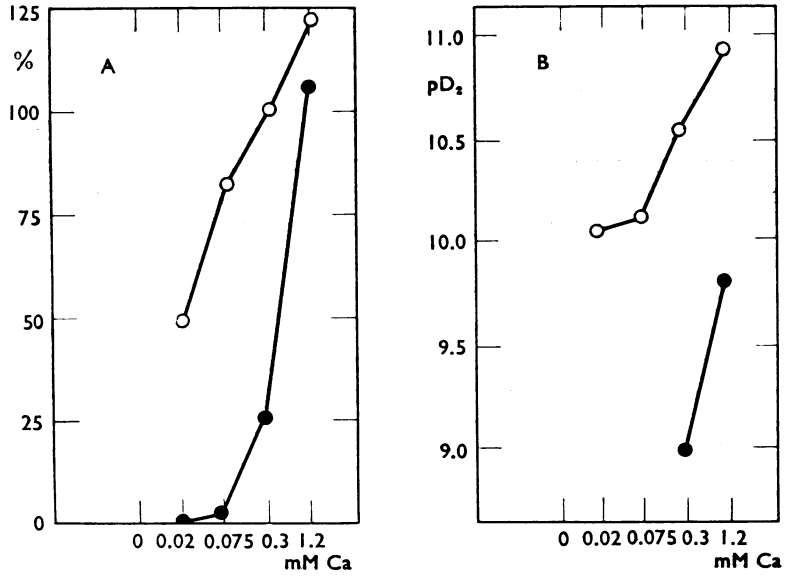

Fig. 5. Effect of the calcium concentration on the response of the rat uterus to oxytocin and methyloxytocin. Oestrogenized rats, Holton's medium, cumulative dose procedure; each point represents the mean of 3–8 experiments. The maximum response achieved (A) is expressed as a percentage of the maximum response to oxytocin in 0.3 mM Ca, the pD_2 (B) is determined by the procedure of Ariëns & van Rossum (1957). ○ oxytocin, ● methyloxytocin. The pD_2 for methyloxytocin in 0.075 mM Ca could not be evaluated because of the very small response.

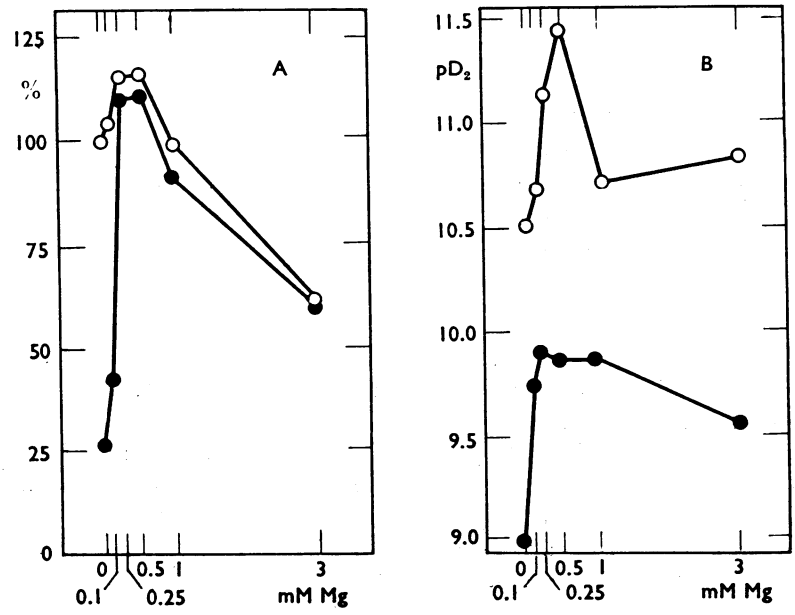

Fig. 6. Effect of the magnesium concentration on the response of the rat uterus to oxytocin and methyloxytocin. Holton's medium with 0.3 mM Ca, other conditions as for Fig. 5. The maximum response (A) is expressed as a percentage of the maximum response to oxytocin in the absence of magnesium, the pD_2 (B) as for Fig. 5.

Effect of increased potassium concentrations

The sensitivity of the uterine strips in depolarizing media as a rule decreased fairly rapidly so that no meaningful dose–response relations could be obtained and no direct comparison of the potency of oxytocin and methyloxytocin could be made. The only demonstrable difference in behaviour was therefore the failure of the analogue in some experiments to cause contraction even in high doses, and its inhibitory effect on the action of oxytocin in such experiments.

In 22 experiments with Krebs-Ringer media the potassium concentration was 47.7, 70, or 100 mM and the calcium concentration was varied from 0.05 to 0.6 mM. The proportion of experiments in which methyloxytocin had a qualitatively oxytocin-like effect, and those in which it inhibited oxytocin, did not vary with the potassium concentration. Inhibition tended to be less frequent at higher calcium concentrations but this trend was not statistically significant.

In K_2SO_4-Ringer media the sensitivity of rat uterine strips was extremely low, and high calcium concentrations were required to obtain response even to high doses of oxytocin (2.5–20 m-u/ml.). In 15 experiments with calcium concentrations between 0.6 and 4.0 mM methyloxytocin acted as an inhibitor of oxytocin activity.

Nature of the antagonism

In many experiments where methyloxytocin inhibited oxytocin, relations characteristic of competitive inhibition were found. An example is shown in Fig. 7 where the log

dose–response curves for oxytocin remain parallel but are displaced along the concentration axis in the presence of the inhibitor; this corresponds to the double reciprocal plots meeting at the origin reported earlier (Rudinger & Krejčí, 1962). However, the values of pA_x (Schild, 1949; Rocha e Silva, 1959) for the antagonistic effect of methyloxytocin calculated from such experiments varied considerably, not only as between different organ preparations but sometimes even in repeated experiments on the same organ. An estimate of the molar ratio of antagonist which caused an approximately 50% reduction in response to a submaximal dose of oxytocin varied from 12:1 to 125:1 with rat uteri, and from 3:1 to 12:1 (occasionally up to 60:1) with rabbit uteri, again sometimes even with a single experiment.

The inhibitory effect is to some extent time dependent. When oxytocin was added to the organ bath together with methyloxytocin the inhibition was weak; when it was added 2–3 min later inhibition was more marked; an interval greater than 5 min caused no further increase in the effect (Fig. 3).

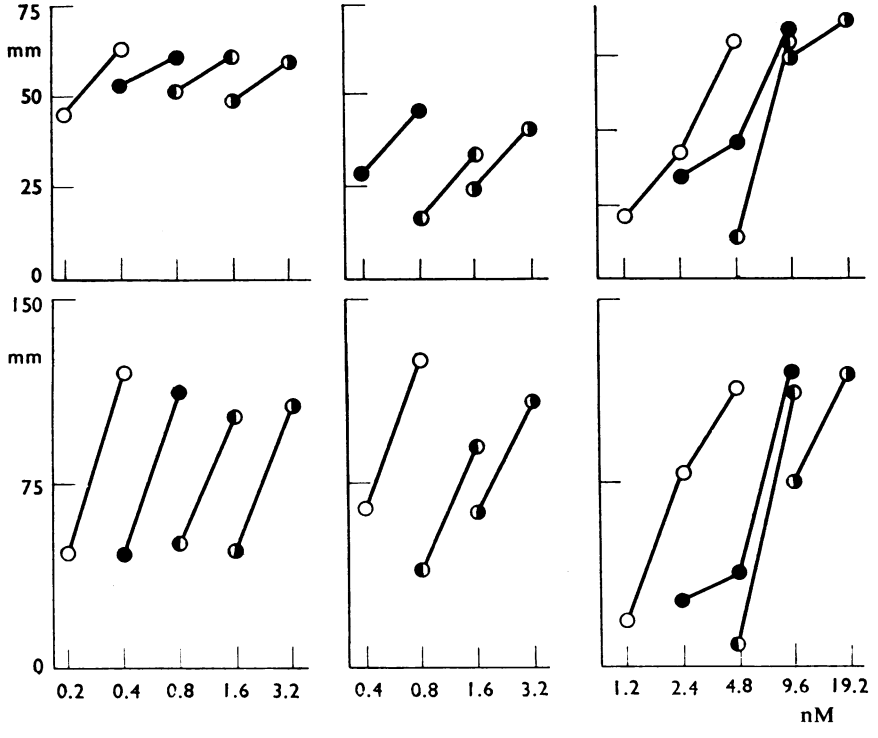

Fig. 7. Effect of methyloxytocin on the response of the rat uterus to oxytocin. Oestrogenized rats, Munsick's medium with 0.2 mM Ca, inhibitor given 3 min before oxytocin. Abscissae: log of oxytocin concentration in n-mole/l. ○ control (no inhibitor); added methyloxytocin, ● 0.1 μg, ◐ 0.2 μg, ◑ 0.5 μg. Each pair of panels shows the results of the experiments on one organ preparation, with the response evaluated either as the height of the most intense contraction (top), or the sum of contractions over 5 min (bottom).

Rat uterus in situ

On the uterus of oestrogenized rats *in situ* (10 experiments) methyloxytocin had a uterotonic effect qualitatively indistinguishable from that of oxytocin as regards, for example, the intensity and frequency of the contractions. The potency was estimated as 20–25 u/mg by the procedure described in the Methods section.

DISCUSSION

The results obtained in this work confirm, extend, and to some degree modify the results published earlier (Rudinger & Krejčí, 1962; Krejčí *et al.*, 1964). It has been confirmed that oxytocin and methyloxytocin differ qualitatively in their action on the isolated rat uterus, and also on the rabbit uterus, over a range of conditions. The change in the effects of methyloxytocin with progressive variation in the experimental conditions can be summarized as a transition from fully oxytocin-like uterotonic action, to stages characterized by decreased frequency and intensity of the rhythmic contractions and a decreased maximal response in cumulative dose experiments. These features become more pronounced until the uterotonic effect disappears and an antagonistic action on the uterotonic effect of oxytocin remains.

One variable affecting the type of response to methyloxytocin is the calcium concentration in the medium. The solutions generally used in organ baths for rat and rabbit uterine strips (Holton, 1948; Munsick, 1960) have lowered concentrations of calcium and little or no magnesium, in order to suppress spontaneous activity. In such media the anomalous properties of methyloxytocin tend to emerge and this tendency is accentuated as the calcium concentration is further decreased (Table 1, Figs. 1 and 3). A second factor is the degree of oestrogenization. On organs taken from animals pretreated with high doses of oestrogen (which are generally more sensitive to oxytocin) methyloxytocin exerts a uterotonic effect, whereas reduced oestrogen dominance favours the appearance of the anomalous properties (Tables 1 and 2) although on organs showing a tendency to spontaneous contractions—whatever their hormonal state—oxytocin and methyloxytocin tend to act alike. Finally, the anomalous behaviour of methyloxytocin is more in evidence at lower temperature.

However, a full, rigorous definition of the conditions under which a particular type of response will reproducibly be obtained is still not possible. This may be due to the operation of other, as yet undefined factors, or to the intrinsic properties of the uterine muscle, which is well known to show a greater variability than many comparable biological preparations.

The consistently oxytocin-like effects of methyloxytocin on the rat uterus *in situ* (an earlier finding of inhibition (Beránková *et al.*, 1961) could not be confirmed) may reflect the *in vivo* values of the relevant variables (temperature, and calcium and magnesium concentrations).

The finding that rat uterine strips contract when exposed to methyloxytocin at least in some depolarizing media makes it unlikely that the membrane potential is the critical factor determining the response to methyloxytocin. It has been proposed that drugs initiate the contraction of uterine muscle by modifying the distribution of calcium in the cell membrane (Csapo, 1961) or, more specifically, by releasing calcium from binding

sites at or near the cell surface. Increased permeability to interstitial calcium is regarded as an alternative (or simultaneous) pathway for calcium penetration (Daniel, Sehdev & Robinson, 1962). In many situations it appears to be surface-bound calcium rather than interstitial calcium which is critical for contraction (Edman & Schild, 1962 ; Daniel, 1963). This may explain some of our findings, too. Thus progesterone increases the stability of calcium binding at the surface sites and this fact has been invoked to explain the spontaneous contractions of progesterone-dominated uteri even at low external calcium concentrations (Berger & Marshall, 1961). The same factor may account for the oxytocin-like action of methyloxytocin on the progesterone-dominated rat uterus, and for the insensitivity of this effect to the external calcium concentration.

The difference in the response to methyloxytocin in the two depolarizing media might be traced to the same cause. In the K_2SO_4-Ringer solution the sulphate ions, by virtue of their high affinity for calcium, could lower the concentration of calcium bound near the cell surface below the level required for the uterotonic action of methyloxytocin.

At the molecular level, the variation in the response to methyloxytocin cannot be due to a mere change in binding. Although the pD_2 values of oxytocin and methyloxytocin (which may be taken as indicative of binding ; Ariëns & van Rossum, 1957) differ, for example, in their dependence on the magnesium concentration the same is also true of analogues showing qualitatively normal behaviour (Bentley, 1965 ; Poláček & Krejčí, unpublished). On the other hand, the maximal response to high doses of methyloxytocin shows a much steeper dependence on the concentration of calcium and magnesium than the corresponding response to oxytocin (Figs. 5 and 6). Furthermore, the ability of methyloxytocin to inhibit the action of oxytocin and, specifically, of structurally closely related compounds (Rudinger & Krejčí, 1962 ; Bisset, 1962) indicates that the analogue retains its ability to interact with the oxytocin receptors. Quantitatively, the excess of methyloxytocin required for inhibition, though rather variable, is of the same order as that required under other conditions to match the uterotonic response to oxytocin. This again suggests that the change in the nature of the response is not in essence due to changes in the affinity of the analogue for the receptor.

The antagonism seemed to be competitive in spite of the variation in pA_x values. The lack of reproducibility may be due to the special properties of the uterus (the rhythmic nature of the contractions, importance of the propagated response, etc.).

According to the occupation receptor theory (for example, Ariëns, van Rossum & Simonis, 1957) a drug retaining its affinity, but having decreased or zero "intrinsic activity," is regarded as forming a drug-receptor complex which is less active in initiating the observable response than the complex formed by the parent drug, or is completely inactive. A transition from agonism to antagonism with changing experimental conditions could be explained by this model—for example, on the assumption that ternary or higher complexes are the active entities, or that the experimental conditions modify the properties of the receptor. The processes linking the hormone–receptor interaction to the final observed effect of muscle contraction are numerous and complex, however, and the simple receptor model can hardly be expected to accommodate all the phenomena observed in this system (Daniel, 1964). Mathematical models can be constructed in which essentially partial agonism at the receptor level can be transformed

to full agonism or to inhibition in the end effect by variation of a parameter in a process intervening between the receptor interaction and the overt effect (V. Pliška, unpublished).

A second feature in the action of methyloxytocin which is difficult to reconcile with the simple receptor occupation model is the time course of some effects—for example, the lower frequency of contractions even under conditions of "full agonism" or the time lag before its inhibitor action becomes fully effective. The superposition of this latter effect on the uterotonic action of methyloxytocin under intermediate conditions presumably accounts for the rapid damping of the contractions initiated by the analogue, and the difference between dose–response curves obtained by the cumulative dose procedure and from individual doses. The times course of inhibition might conceivably be interpreted in terms of the receptor rate theory of Paton (1961); however, the rhythmic nature of the contractions precludes a quantitative analysis of the results in these terms.

SUMMARY

1. The effect of 2-O-methyltyrosine-oxytocin (methyloxytocin), a synthetic analogue of oxytocin, on the isolated rat and rabbit uterus varies over a range of experimental conditions from a qualitatively oxytocin-like but relatively weak uterotonic action, through responses characterized by decreased frequency and diminishing amplitude of the contractions and a decreased maximal response to saturation doses, to complete loss of activity and inhibition of the uterotonic action of oxytocin. The inhibition is reversible and often appears competitive.

2. The anomalous properties of the analogue are found more frequently with oestrogen-dominated than with progesterone-dominated uteri and they are favoured by low concentrations of calcium in the medium, by the absence of magnesium, and by low temperature. Methyloxytocin may also act as an antagonist of oxytocin on the depolarized uterine strip. On the uterus *in situ* the analogue exerts an oxytocin-like effect.

3. The possibility is discussed that the amount of membrane-bound calcium is of importance in determining the character of the response to methyloxytocin.

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