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NEUROHYPOPHYSIAL PEPTIDE INTERACTION WITH MAGNESIUM IN AVIAN VASCULAR SMOOTH MUSCLE*

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SUMMARY

1. The contractile responses of helically cut vascular strips of chickens to vasoactive agents were studied.

2. Large pulmonary arteries were contracted by neurohypophysial peptides, but not by angiotensin, acetylcholine, histamine, 5-hydroxytryptamine, bradykinin or eledoisin. The activity of oxytocin was greater than that of arginine vasopressin.

3. Vasodilator effects of oxytocin upon small (200–500 μ diameter) mesenteric and muscular arteries were demonstrable, but inconsistent.

4. Magnesium potentiated in a parallel fashion the vasoconstrictor effects of oxytocin and of arginine vasopressin.

5. Deamino-oxytocin was potentiated by magnesium. This finding suggests a difference between the peptide-protein interactions of tissue receptors and those of neurophysin.

INTRODUCTION

In previous papers we showed that magnesium potentiates the response of several mammalian contractile tissues to neurohypophysial peptides (Somlyo, Woo & Somlyo, 1966; Woo & Somlyo, 1967). We further described a vasodilator action and a relaxant action on tracheal smooth muscle of chlorobutanol, the preservative contained in commercial oxytocin preparations. The preservative also has demonstrable vasodepressor activity *in vivo* (Katz, 1964) and relaxes isolated intestinal smooth muscle (Axelsson, Holmberg & Högberg, 1965). These findings suggested that some of the inhibitory actions ascribed to oxytocin may have been

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due to the chlorobutanol contained in commercial preparations. However, those *in vivo* studies utilizing preservative-free posterior pituitary or synthetic preparations (Hogben & Schlapp, 1924; Sawyer, 1961) gave clear evidence of the vasodepressor activity of these polypeptides, and we had previously demonstrated the intestinal relaxant effects of preservative-free oxytocin and vasopressin *in vitro* (Woo & Somlyo, 1967).

The purpose of the present study was to determine whether magnesium potentiates the action of oxytocin, vasopressin and their analogues in birds, as it does in mammals. In order to determine whether the previously proposed Mg-facilitated binding of neurohypophysial peptides to receptors of contractile tissues (Somlyo, Woo & Somlyo, 1966; Woo & Somlyo, 1967) is mediated at sites involved in other peptide-protein interactions, we also wanted to examine the effects of Mg upon the biological activity of deamino-oxytocin. This analogue, unlike oxytocin and vasopressin, is not bound to neurophysin, the peptide-binding protein isolated from posterior pituitary glands (Hope, 1964; Breslow & Abrash, 1966). Our second objective was to demonstrate, if possible, the vasodilator effect of preservative-free oxytocin on isolated vascular smooth muscle. We have selected the domestic fowl for these studies, because of the known great sensitivity of this species to oxytocin, which has been utilized by Coon (1939) for the development of the standard avian depressor assay of neurohypophysial peptides.

The following report includes an unexpected finding of some interest: the specific sensitivity of isolated avian pulmonary arteries to the vasoconstrictor action of oxytocin and related peptides.

METHODS

A total of twenty-seven domestic chickens, weighing 1·6–4·1 kg, was used. The majority of animals (15) used were roosters, because of the known effects of estrogen upon the vascular response to oxytocin in mammals (Lloyd & Pickford, 1962). However, we detected no noticeable difference between the vascular responses of the two sexes. The animals were killed by decapitation. Helically cut strips of large vessels were obtained in the manner previously reported (Somlyo & Somlyo, 1964; Somlyo *et al.* 1965). The initial load placed on strips of the right and left (primary branches) pulmonary arteries was 2 g, on strips of pulmonary veins 100–600 mg. Small vessel (200–500 μ diameter) segments were removed from small pulmonary, mesenteric and muscular arteries. The segments were slipped over a stainless-steel stylet (150 μ diameter) and helical strips, approximately 1 cm long, were cut with ophthalmological scissors under a stereomicroscope. Strips obtained from small vessels were placed under tension (100–200 mg) by stretching them approximately 45% above the excised length.

All solutions were prepared with distilled, deionized water. The composition of Krebs solution, aerated with 95% O_2 and 5% CO_2, has been reported previously (Woo & Somlyo, 1967). This solution contains 1·2 mM Mg, which was omitted from the Mg-free Krebs solution. Solutions with other Mg concentrations were prepared by adding appropriate amounts of $MgSO_4$ to the Mg-free solution. All experiments were conducted at 40–42° C. The isotonic

and isometric recording methods employed in our laboratory have been described in detail (Somlyo & Somlyo, 1964; Somlyo *et al.* 1965). When shortening was expressed in mm/cm resting length, the latter was the length of the freshly prepared vascular strips (Somlyo, Sandberg & Somlyo, 1965).

The following drugs were used: preservative-free synthetic oxytocin, 430 oxytocic u./ml. (Sandoz, Lot 64004); bovine arginine vasopressin powder, 52·2 rat pressor u./mg, 2·1 oxytocic u./mg (Parke, Davis and Co., Lot 274927) diluted, without preservative, as described previously (Woo & Somlyo, 1967); the following synthetic peptides used were made available to us by the manufacturer (Sandoz Laboratories) in ampoules containing chlorobutanol (5·0 mg/ml.) as the preservative: 1-deamino-oxytocin 0·7 u./ml. (avian depressor activity) (ODA 914, bradykinin 1·0 mg/ml. (BRS 640), and eledoisin 0·07 mg/ml. (ELD 950). (−)-noradrenaline bitartrate powder (Sigma) was dissolved in distilled, deionized water, with a minimum amount of 0·1 N-HCl; histamine acid phosphate (Burroughs Wellcome), acetylcholine chloride (Merck and Co., Inc.), 5-hydroxytryptamine creatinine sulphate (Sigma) were dissolved in 0·85 % NaCl. Angiotensin amide (Ciba) and metaraminol (Aramine) were available in ampoule form. Chlorobutanol 0·5 % was available as the diluent used for Syntocinon (R).

Statistical significance was evaluated by the conventional Student *t* test (Alder & Roessler, 1962).

RESULTS

Responses of large pulmonary artery, pulmonary vein, and sciatic artery to oxytocin and vasopressin. Strips obtained from pulmonary arteries and veins were invariably sensitive to the vasoconstrictor action of oxytocin.

Oxytocin cumulative log dose–response curves of pulmonary artery strips shown in Fig. 1*A* indicate a threshold concentration of approximately 0·05 m-u./ml. oxytocin, and an ED 50 of approximately 1·5 m-u./ml. in normal Krebs solution. The sensitivity of the fowl pulmonary artery preparation to arginine vasopressin under identical conditions (Fig. 1*B*) is considerably less, when calculated on a molar basis. If the vasopressin concentrations, expressed in rat pressor units in Fig. 1*B*, are converted to oxytocic units (2·1 oxytocic/52·2 rat pressor) the threshold and ED 50 pulmonary vasoconstrictor concentrations are similar to those required with oxytocin in the cumulative dose–response curves.

The maximal responses of fowl pulmonary artery strips to vasopressin (Fig. 1*B*) appeared less than those to oxytocin, but the difference between means of this small series was not statistically significant ($P > 0{\cdot}05$). In two experiments, after the response to vasopressin had reached a plateau, the addition of oxytocin to the bath produced a further, small contraction, suggesting that the differences in maximal responses to the two peptides were real.

Pulmonary vein strips (four log-dose–response curves) appeared as sensitive as the arteries to the vasoconstrictor action of oxytocin.

Sciatic artery strips were either completely insensitive (five preparations) or very slightly responsive to the vasoconstrictor action of oxytocin (four preparations). The maximal responses of the latter were less than $\frac{1}{2}$ of the

maximal responses of the same strips to noradrenaline (Somlyo & Woo, 1967). Similar large differences in the maximal responses of mammalian vascular smooth muscle to different drugs have been described and possible mechanisms discussed elsewhere (Somlyo *et al.* 1965; Somlyo & Somlyo, 1966*a*, *b*).

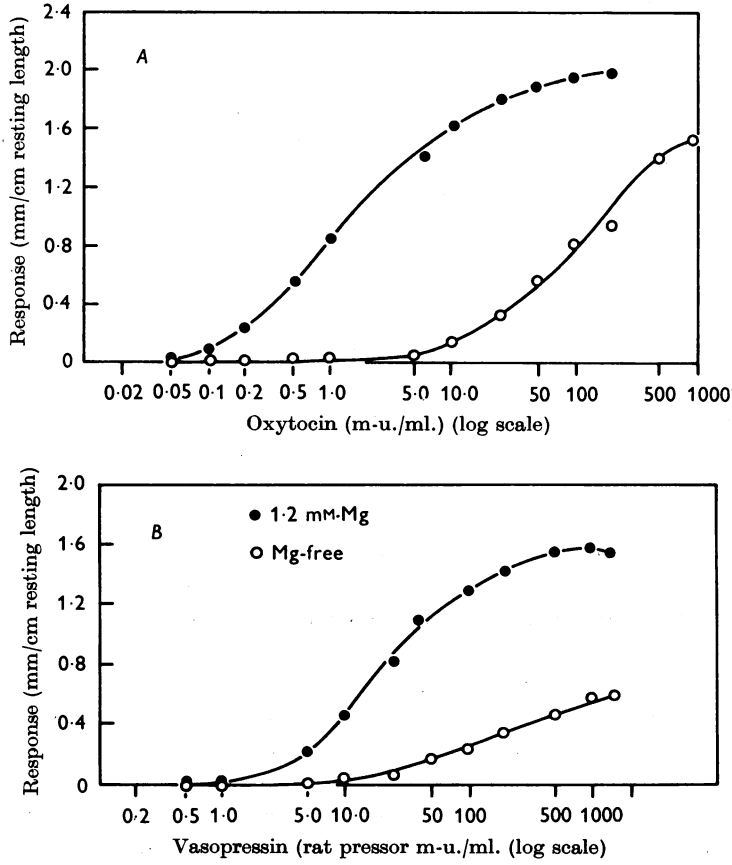

Fig. 1. Neurohypophysial peptide action on strips of fowl large pulmonary arteries: effect of Mg. Cumulative log dose–response curves to oxytocin and to arginine vasopressin (isotonic contractions). In *A* each point represents mean response to oxytocin of 12–15 strips (in Krebs solution) and 6–8 strips (Mg-free solution). Vasopressin log dose–response points (*B*) are mean responses of five strips. For description see text.

Acetylcholine, histamine, 5-hydroxytryptamine, angiotensin, bradykinin and eledoisin: effects on strips of large pulmonary arteries. The great sensitivity of this preparation to oxytocic peptides suggested to us that the fowl pulmonary artery may be a useful source of bio-assay material. We have

therefore determined the specificity of the responses of this preparation to a variety of vasoactive agents. The results obtained with noradrenaline have been published separately (Somlyo & Woo, 1967). None of the other compounds studied (Table 1) had vasoconstrictor activity on fowl large pulmonary arteries, with the exception of a single, very slight response to a high concentration of 5-hydroxytryptamine (16·7 μg/ml), not included in Table 1.

TABLE 1. Agents lacking vasconstrictor activity on (large) fowl pulmonary arteries

Drugs	Concentration (μg/ml.)	No. strips
Angiotensin	10·0–15·0	9
5-Hydroxy-tryptamine	0·8–10·0	11
Acetylcholine	10·0	6
Histamine	10·0	6
Bradykinin	10·0	9
Eledoisin	0·7	9

Angiotensin (10·0 μg/ml.), in particular, had no vasoconstrictor activity, even on sciatic artery preparations, thus supplementing the *in vivo* studies which suggest that angiotensin has no direct pressor activity in chickens (Harvey, Copen, Eskelson, Graff, Poulsen & Rasmussen, 1954).

The kinin preparations used in these experiments contained chlorobutanol (Methods). In those species whose pulmonary vessels are reported to be constricted by bradykinin or eledoisin of the same manufacturer, these peptides are highly active at very low concentrations (Kovalcik, 1963; Moog & Fischer, 1964; Hauge, Lunde & Waaler, 1966). It is unlikely, therefore, that the chlorobutanol delivered (50·0 μg/ml.) with the maximal concentrations of kinins indicated in Table 1 would have completely masked significant vasoconstrictor activity of these peptides, if present.

Magnesium and the pulmonary vasoconstrictor action of neurohypophysial peptides. Oxytocin and vasopressin dose–response curves were, in Mg-free solution, shifted by approximately two log units to the right of the respective curves obtained in normal Krebs (1·2 mM-Mg) solution (Fig. 1*A* and *B*). Inspection of these dose–response curves suggests that if the intrinsic activity of vasopressin was in fact lower than that of oxytocin, this could create the appearance of greater Mg-dependence of the less active peptide.

The Mg-dependence of oxytocin and arginine vasopressin was therefore studied in greater detail, in the manner previously employed in a study of canine vascular smooth muscle (Somlyo *et al.* 1966). In Fig. 2 are plotted the isotonic responses of pulmonary artery strips to fixed doses of oxytocin (1 m-u./ml.) and to vasopressin (5 m-u./ml.), at different Mg concentrations. It is apparent that the shapes of the curves obtained with

oxytocin and vasopressin are similar. These results do not support the suggestion (Munsick, 1960) that the potentiating action of Mg is greater with vasopressin than with oxytocin. The significance of the inflexion in Fig. 2, between 1·2 and 4·8 mM-Mg, is unknown, and may reflect cation-induced effects independent of its potentiating action. The variability of the responses of different smooth muscles to the direct effect of Mg has been reported (Woo & Somlyo, 1967).

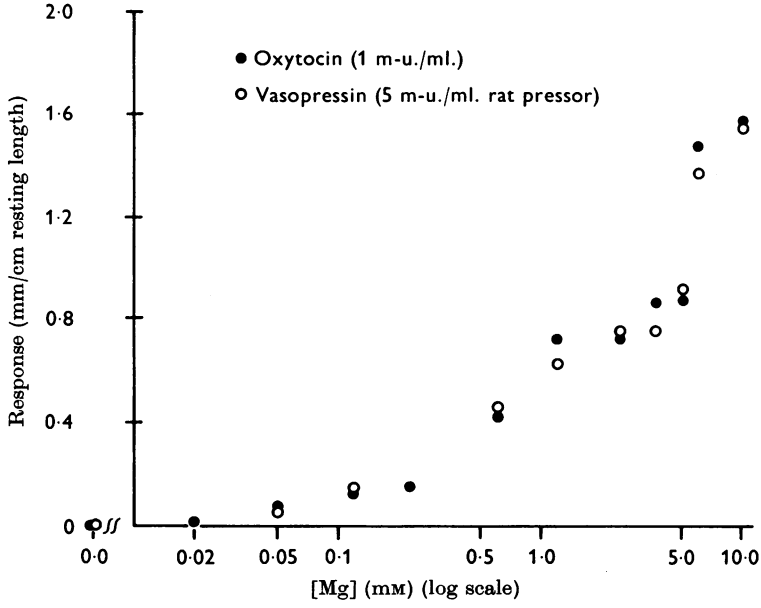

Fig. 2. Mg-dependence of oxytocin and of arginine vasopressin. Isotonic responses of fowl large pulmonary artery strips to fixed doses of oxytocin (1 m-u./ml.) and of arginine vasopressin (5 m-u./ml.), at different Mg concentrations (abscissa). Each point represents mean isotonic responses to oxytocin (7–11 strips) or vasopressin (5–9 strips).

Deamino-oxytocin and the potentiating action of Mg. Preliminary log dose–response curves of the action of deamino-oxytocin (ODA) on pulmonary artery strips indicated the potentiating effect of Mg upon this peptide. We subsequently studied paired strips, one placed in normal (1·2 mM-Mg) Krebs and the other in Mg-free solution. The preparations in Mg Krebs were contracted by ODA, while the strips bathed in Mg-free solution were almost completely unresponsive. It was not feasible, with the ODA preparation available to us, to obtain valid dose–response curves in Mg-free solution, because these ODA ampules also contain approximately 7·1 μg chlorobutanol for each m-u. of ODA. The vasodilator activity of chlorobutanol (see below) would reach prohibitively high levels, inhibiting the vasoconstrictor action of ODA.

When ODA was added to a strip in Mg-free solution (Fig. 3), the addition, during the plateau-phase of contraction, of 10 mM-Mg produced a secondary contraction within 13–30 sec (by stopwatch). Mg added in this manner to a strip contracted with noradrenaline or to one resting in Mg-free solution had no contractile effect.

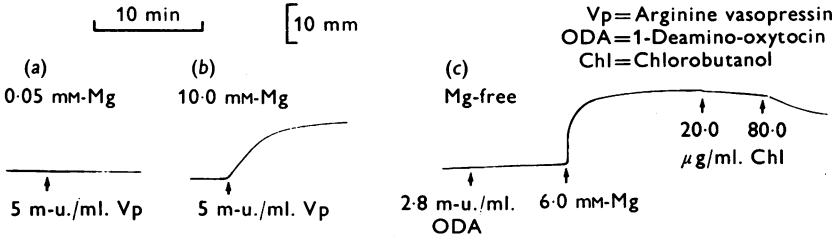

Fig. 3. Potentiation of deamino-oxytocin and arginine vasopressin by Mg. Isotonic contractions of a fowl large pulmonary artery strip. Note difference in response to same concentration of vasopressin at: (*a*) low and, (*b*) high Mg concentrations. After (*b*) strip placed for ½ hr in Mg-free solution and (*c*) its response to deamino-oxytocin was determined. Note that the minimal response to peptide is followed by marked secondary contraction in 16 sec (by stop watch) after adding Mg to bath. Chlorobutanol produced moderate relaxation of contracted strip.

In five experiments, we determined responses to fixed concentrations of ODA at different Mg-concentrations, in the manner shown in Fig. 2. These experiments also confirmed the potentiating action of Mg (0–10 mM) upon ODA.

Responses of strips of small vessels to oxytocin and to chlorobutanol. The vasoconstrictor action of oxytocin was generally much more readily demonstrable than the vasodilator action. To demonstrate a vasodilator effect, before the addition of oxytocin, the majority of the systemic artery strips were preconstricted with noradrenaline, 5-hydroxytryptamine or metaraminol. Pulmonary artery strips were preconstricted with 5-hydroxytryptamine. The responses of small vessel preparations to oxytocin were also determined under resting tone, without preconstriction.

Pulmonary artery strips from vessels 200–500 μ in diameter were contracted by oxytocin (Fig. 4), and we did not demonstrate a vasodilator or diphasic effect with these preparations. There was no evidence of autoinhibition due to some inhibitory effect of oxytocin: the maximal responses to the peptide were equal to the maximal responses of the same strips to 5-hydroxytryptamine. Noradrenaline, in concentrations up to 32·0 μg/ml., had either no effect or relaxed small pulmonary arteries of the fowl (five experiments).

Of the systemic small vessels studied (eight mesenteric and seven muscular arteries), six gave diphasic responses (Fig. 4) to oxytocin. In these preparations relaxation preceded contraction following addition of one

or more of the cumulative doses of oxytocin (0·3–180 m-u./ml.) during determination of the dose–response curves. Vasodilation of strips pre-contracted with 5-hydroxytryptamine, metaraminol or noradrenaline could be demonstrated in only six out of sixteen experiments on systemic small vessels.

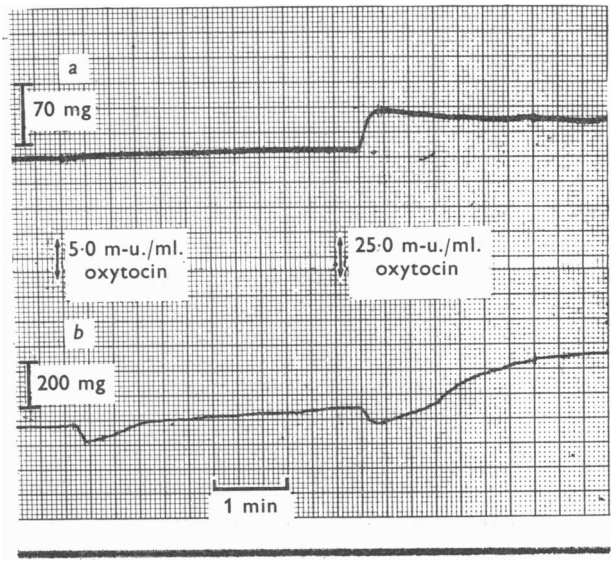

Fig. 4. Effect of oxytocin on small fowl vessels. Isometric responses of two preparations in same muscle bath. (*a*) small pulmonary and, (*b*) small mesenteric artery strip. For further details, see text.

In a few experiments, various methods were tried to accentuate the inconsistent vasodilator action of oxytocin, without noticeable success. These unsuccessful manipulations included the use of chicken plasma or epinephrine (Haigh, Lloyd & Pickford, 1965) to preconstrict the vascular strips.

The maximal isometric contractions elicited by noradrenaline and by 5-hydroxytryptamine in mesenteric and muscular arteries, as determined from cumulative dose–response curves, were equal. In contrast, the maximal response of the same preparations to oxytocin was only 50 % of the response to the amines. Chlorobutanol (10·0–100·0 μg/ml.) relaxed each of the six mesenteric and muscular small artery preparations studied.

DISCUSSION

The vasodilator action of oxytocin upon small avian mesenteric and muscular arteries provides some confirmatory evidence for the direct inhibitory effects of this peptide, although this effect in our experiments

was very inconsistent. We were unable to determine the factors which allowed us to demonstrate relaxation of a few vascular strips by oxytocin or prevented this effect in the majority of preparations. This was not due to the strain of chickens used, since intact, anaesthetized chickens obtained from the same supplier exhibited the characteristic systemic vasodepressor response to intravenous oxytocin (unpublished observation). According to work in Pickford's laboratory (Lloyd & Pickford, 1962) sympathectomy converts the vasodepressor effects of oxytocin to a vasopressor one in several mammalian species. Woolley & Waring (1958), working with isolated chicken legs, found that acute division of the sciatic nerve did not interfere with oxytocin-induced vasodilation. A similar, but more drastically separated fowl-leg preparation reported in the same study was not sensitive to the vasodilator action of oxytocin. It is possible that the responsive preparation contained intact sympathetic fibres or that the different time intervals elapsing between sympathectomy and the injection of oxytocin were responsible for the variations in responsiveness. A similar interpretation may apply to our isolated preparations which were removed from their nerve supply 1–3 hr before exposure to oxytocin. However, we cannot rule out the possibility that arterioles are more responsive to the inhibitory effects of oxytocin than the small arteries used in our experiments. A certain specificity of the vasodilator effect may be inferred from the fact that none of the strips from large or small pulmonary arteries could be relaxed by oxytocin.

None of the vascular preparations studied was relaxed by oxytocin without also exhibiting a contractile response. The pronounced vasoconstrictor action of oxytocin upon pulmonary vessels, unaccompanied by vasodilation, was unexpected. Assuming that the avian vasopressor activity of pure oxytocin is approximately 500 u./mg (Van Dyke, Sawyer & Overweg, 1963), the effective threshold concentration observed in 1·2 mM-Mg Krebs solution (0·5 m-u./ml.), was 10^{-10} M, and the ED 50 (1·5 m-u./ml.) 3×10^{-9} M. In comparison, assuming that the rat pressor activity of bovine arginine vasopressin is approximately 400 u./mg, the threshold (1 m-u./ml.) and ED 50 (20 m-u./ml.) of this peptide were, respectively, $2{\cdot}5 \times 10^{-9}$ and 5×10^{-8} M. It is becoming evident that there exists a group of blood vessels, which are more sensitive to the vasoconstrictor action of oxytocin than to that of vasopressin. These include human umbilical vessels (Somlyo *et al.* 1965) and, judging from our present study, the vasculature of the domestic fowl.

The high degree of specificity of large pulmonary arteries of chickens to the contractile action of neurohypophysial peptides commends this preparation for further studies on mechanisms of hormone action, and perhaps for use as an ancillary bioassay. In addition to its sensitivity and relative

specificity, the pulmonary artery strip is not spontaneously active and responds with graded contractions at body temperature. For this reason, we have already used it to determine the interaction of Mg with neurohypophysial peptides in avian vascular smooth muscle.

Magnesium potentiated the contractile action of neurohypophysial peptides upon avian vascular smooth muscle, extending to this class of vertebrates the generality of Mg-peptide interaction (Somlyo *et al.* 1966; Woo & Somlyo, 1967). This interaction is already present in vascular smooth muscle of the Pacific hagfish (Somlyo & Somlyo, 1967), one of the earliest evolutionary forms known to possess oxytocin-like hormones. We have reviewed elsewhere (Somlyo *et al.* 1966; Woo & Somlyo, 1967) the evidence for the suggested mode of potentiating action of Mg: this cation causing an increased affinity between neurohypophysial peptides and the receptors of contractile tissues. The potentiating action of Mg upon both the inhibitory and the excitatory action of vasopressin on intestinal smooth muscle (Woo & Somlyo, 1967) also suggests that the cation affects an early step of peptide-muscle interaction. These considerations, as well as the dual effect of oxytocin upon some of our avian small vascular strips, suggest that two different messengers, rather than two receptors for attachment, mediate respectively the relaxant and contractile effects of neurohypophysial peptides. Vasopressin has a somewhat similar dual, inhibitory followed by excitatory, effect upon isolated rabbit colon rings (Woo & Somlyo, 1967), but with the intestinal preparation, the possibility could not be ruled out that inhibitory and excitatory responses originated from different muscle layers.

Deamino-oxytocin is not bound to either crude neurophysin or to the purified fractions of this peptide-binding pituitary protein (Hope, 1964; Breslow & Abrash, 1966). According to most current theories of drug-tissue interaction (Furchgott, 1964), the biological activity of this peptide implies that it is bound to receptors of smooth muscle. Two previous studies noting the effect of Mg upon the uterine effects of deamino-oxytocin came to apparently conflicting conclusions (Chan, O'Connell & Pomeroy, 1963; Munsick & Jeronimus, 1965). Some of these differences may have been due to the pharmacological custom of expressing potency as the function of a standard pituitary preparation (Munsick, 1960). Measuring biological activity in this manner, if Mg were to potentiate equally the responses to some unknown peptide and to the standard employed, one would reach the conclusion that Mg does not increase the 'potency' of that given peptide. Our experiments provide a clear cut demonstration that the action of deamino-oxytocin upon vascular smooth muscle is potentiated by Mg in a manner similar to vasopressin and oxytocin. Thus, Mg does not appear to exert its effect at the protonated site, required for binding by

neurophysin, which is absent in deamino-oxytocin (Hope, 1964; Breslow & Abrash, 1966).

Finally, we want to call attention to two technical considerations related to these studies. First, it appears that the use of Mg-free solutions may obscure certain Mg-dependent effects of neurohypophysial peptides. A peptide which has inherently lower intrinsic activity or efficacy in a given system would be affected more by lack of the potentiating cation than a more active peptide. This is to be expected if current models of receptor theory and spare receptors have any validity (Furchgott, 1964). A second technical consideration involves the presence of chlorobutanol in several commercial peptide preparations, including oxytocin and vasopressin analogues as well as kinins. The biological activity of chlorobutanol is far from negligible, and may have been a source of error in some previous studies on neurohypophysial peptides. Thus, we now question whether the reputed vasodilator activity of synthetic vasopressin analogues (Somlyo *et al.* 1965) was due to chlorobutanol. Contamination by this compound is, of course, greatest in preparations whose concentration of the peptide itself, such as deamino-oxytocin, is relatively low. If the latter preparation is biologically assayed in low Mg solutions (Munsick, 1965), its potency may appear to be lower than that of preservative-free deamino-oxytocin (Chan *et al.* 1963).

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