Effect of Mg^{2+} on the binding of oxytocin to sheep myometrial cells

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Binding isotherms of oxytocin to sheep myometrial cells in a short-term cell culture were investigated in the presence of Mg^{2+} in the concentration range 0–10 mM. The occurrence of at least three binding sites had been demonstrated earlier. Mg^{2+} influences individual sites and individual features of the binding isotherm differently. Dissociation constants (K_d) of the high- and medium-affinity sites attain a minimum of 6.8×10^{-10} and 4.2×10^{-8} mol/l respectively at 2.75 mM- Mg^{2+} . The two sites display the highest binding capacity (B) at 1 mM- Mg^{2+} (ratio of high affinity/medium affinity is 1:16). The B/K_d quotients reflecting relative binding (bound-to-free concentration) at the half-saturation of binding sites also have their maxima at 1 mM- Mg^{2+} . The high-affinity site displays a strong positive co-operativity (Hill coefficient at 4 mM- Mg^{2+} of 2.4), which is amplified in the presence of Mg^{2+} . Positive co-operativity of the medium-affinity site is markedly lower (Hill coefficient at 4 mM- Mg^{2+} of 1.5) and shows less dramatic Mg^{2+} -dependence. Low-affinity sites are not co-operative at any Mg^{2+} concentration. It is concluded that Mg^{2+} may display its effect upon the oxytocin–receptor interaction predominantly by influencing positive co-operativity.

INTRODUCTION

The sensitivity of the rat uterus to oxytocin *in vitro*, expressed as a negative logarithm of the half-maximal dose (pD_2), is influenced in a non-monotonic manner by the Mg^{2+} concentration in the organ medium (Munsick, 1960; Krejčí *et al.*, 1964; Bentley, 1965; Munsick & Jeronimus, 1965; Krejčí & Poláček, 1968). The response is regularly increased by Mg^{2+} at a concentration range from zero up to roughly 0.5 mM, whereas concentrations above this value cause attenuation of the response (Krejčí *et al.*, 1964; Krejčí & Poláček, 1968). Structural analogues of oxytocin sometimes display considerable deviations from this behaviour: Mg^{2+} at 0.5 mM may both potentiate and antagonize the effect of a peptide agonist (as compared with the Mg^{2+} -free solution), and the ' pD_2 -quotient', defined as pD_2 at 0.5 mM- Mg^{2+} minus pD_2 in Mg^{2+} -free solution, is positively correlated with the potency of the analogue (Krejčí & Poláček, 1968; Soloff & Grzonka, 1986).

The effect of Mg^{2+} cannot be ascribed to a certain phase of the uterotonic response, since pD_2 , as a potency descriptor, reflects kinetic parameters of manifold processes participating in this response, such as hormone–receptor interaction, transduction (G-protein, enzymic steps, etc.) or the muscle contraction itself (Pliška, 1988). However, when considering the differences among the structural analogues mentioned above, it seems that the observed effect of Mg^{2+} is likely to be associated, at least in part, with the peptide–receptor interaction. An effect of Mg^{2+} upon the binding of oxytocin to its putative myometrial receptor has been described, in terms of overall binding at a single ligand concentration, by Soloff & Swartz (1974). The bound fraction in these experiments, in which a single (low) oxytocin concentration was used, displayed a several-fold increase with increasing Mg^{2+} concentrations of up to 10 mM. More recently, Antoni & Chadio (1989) found a positive Mg^{2+} effect upon the affinity of oxytocin for its receptor in rats, and Ayad & Wathes (1989) described an effect of this cation on maximum oxytocin binding in sheep.

Previous investigations have demonstrated the existence of

several binding sites on rat, sheep (Pliška *et al.*, 1986; Crankshaw *et al.*, 1990), and perhaps also human (Lopez Bernal *et al.* 1989), myometrial cells. Previous studies of the Mg^{2+} effect considered only a single, high-affinity, binding site for oxytocin which, in our opinion, may not represent the true receptor (Pliška, 1988). In this context, questions arise as to the extent of the Mg^{2+} effect upon the binding of oxytocin to individual sites, and as to possible similarities between the effect on binding and the effect upon response descriptors such as pD_2 and/or the maximal contractile response of the uterus. We have approached this problem by carrying out binding experiments enabling us to estimate relevant binding parameters at several Mg^{2+} concentrations. In order to avoid uncertainties which might be associated with the use of membrane fractions in previously reported experiments, we used a monolayer culture of sheep myometrial cells.

MATERIALS AND METHODS

Culture of sheep myometrial cells

Myometrial cells were obtained from the uteri of young ewes (4–6 months of age: predominantly White Alpine Sheep). The procedure followed was virtually identical to the protocol of Pliška *et al.* (1986), which aims at the removal of a large proportion of fibroblasts by pre-plating (discarding cells which adhere on the surface of the culture flask within 1 h). The number of cells per unit volume was estimated using a cell counting chamber. On average, 95 % of the cells did not stain with Trypan Blue and were thus considered to be viable. Protein concentration was measured by an adaptation of the method of Lowry *et al.* (1951), as indicated previously (Pliška *et al.*, 1986).

Immunostaining of the cultured cells with a monoclonal antibody against desmin (Boehringer Mannheim, product no. 814377; dilution 1:50) by the procedure of Debus *et al.* (1983) indicated the presence of about 80 % smooth muscle cells, compared with 30 % in the quickly adhering cell fraction (H. Kohlhauf Albertin & V. Pliška, unpublished work).

Abbreviations used: pD_2 , negative logarithm of half-maximal dose; c_b , bound ligand concentration; c_f , free ligand concentration; B , binding capacity.

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Peptides

Oxytocin, ^3H -labelled at positions 3 and 5 of Tyr-2 was a product of Amersham International, Cardiff, U.K., and was purified on a reversed-phase h.p.l.c. The specific radioactivity was approx. 29 Ci/mmol. The specific radioactivity of working solutions was adjusted to 6–29 Ci/mmol by dilution with unlabelled oxytocin (Ferring AB, Malmö, Sweden).

Binding experiments

Equilibrium binding experiments were carried out as described previously (Pliška *et al.*, 1986). Cells (mean 1.4×10^6 cells/ml) were incubated in Hepes-buffered physiological salt solution with 2.5×10^{-10} to 2.5×10^{-5} M [^3H]oxytocin at 22 °C for 60 min. The standard incubation volume was 200 μl ; in order to keep random errors low, this volume was increased up to 5 ml (by keeping the number of cells/ml constant) when c.p.m. in the bound fraction were lower than 500 (in cases of isotopically diluted ligand, i.e. for high total ligand concentrations). MgCl_2 concentration was 0–10 mM. Incubation was interrupted by cooling on ice, and the cells were collected by rapid centrifugation in an Eppendorf centrifuge, rinsed with the corresponding Mg^{2+} -containing buffer and dissolved overnight in 50 μl of Tissue-Sol (C. Roth G.m.b.H., Karlsruhe, Germany) for β -radiation counting; radioactivity was converted into bound ligand concentration (c_b). Portions of supernatant were counted for radioactivity and resulting d.p.m. values were converted into free ligand concentrations (c_f).

Binding isotherm and data fit

The superimposed form of the binding isotherm consisting of several Hill terms (Pliška *et al.*, 1986) is as follows:

$$c_b = \sum_i c_{b,i} \quad (1)$$

where:

$$c_{b,i} = B_i / [(K_{d,i}/c_f)^{h_i} + 1] \quad (2)$$

with B_i , $K_{d,i}$ and h_i being the binding capacity, the dissociation constant of the oxytocin-binding site complex and the Hill coefficient of the i th site respectively. This isotherm was fitted to the c_f and c_b data. Binding capacities, dissociation constants and the Hill coefficients were estimated by the modified affinity spectra procedure (Tobler & Engel, 1983) described previously (Pliška *et al.*, 1986). For a particular group of $\{c_f, c_b\}$ data (12–36 pairs in our experiments), the method delivers a set of g triplets $\{B_m, K_{d,m}, h_m\}$ estimated from ascendantly ordered partial sequences of n -pairs $\{c_f, c_b\}$. The number of these triplets is:

$$[(n - n_{\min.} + 1)(n - n_{\min.} + 2)]/2 \quad (3)$$

where $n_{\min.}$ is the length of the shortest data sequence employed in computations ($n_{\min.} = 5$ in our case). (The actual n is smaller in most of the calculations, since the fit aborts for certain sequences.) The h value was estimated for each partial sequence by means of an optimization routine (Fibonacci fit) described previously (Atkins, 1973). This value is employed for transformation in $\{c_b, c_b/c_f^{h_i}\}$ co-ordinates, the so-called 'linearized Scatchard plot' (Pliška *et al.*, 1986), which yields a linear correlation in cases where the sequence is nested around a single binding site. Further evaluation of the spectra is then based on parameter triplets, yielding the coefficient of linear correlation (r) of the linearized Scatchard plot that is significant at a certain probability level (usually 1 %).

The parameter estimates were then improved by means of a non-linear Gauss-Newton algorithm applied to eqn. (1) consisting of two to four terms given by eqn. (2); the number of

terms equalled the number of sites identified by the affinity spectra method. Weighted means of the three parameters were taken as initial estimates. The weight S_r was defined as:

$$S_r = \log [(2 - P)/P], \quad (4)$$

where P is the estimated significance of r ($0 \leq P \leq 1$); S_r values are from the interval $0 \leq S_r \leq \infty$. A corresponding non-linear regression routine from the BDMP Statistical Software (Dixon, 1983) was employed. In most cases, however, the improved values deviated by only a small degree from the original estimates (except for some binding data for which the affinity spectra method yielded rather uncertain clusters). The evaluation procedure is exemplified in Figs. 1 and 2.

The binding experiment was repeated four to seven times for each Mg^{2+} concentration. Affinity spectra indicated two to three significantly different clusters consisting of data for individual partial sequences. The resolution of the spectrum depends largely on the ligand concentration interval used in the binding studies. In several instances, some data, particularly at the lower limit of this interval, were outliers (due to a large error when measuring too low c.p.m. of bound ligand). Thus single binding isotherms may not cover all binding sites, and the number of estimates for individual points may differ (cf. Figs. 4 and 5).

RESULTS

Identification of binding sites

The results of the analysis of binding data obtained with 4.25 mM- Mg^{2+} (optimum for the high-affinity binding sites, see below) are shown in Fig. 1. Each vertical line in the spectrum (Fig. 1b) indicates a single K_d estimate (abscissa) and its probability, as mentioned above (ordinate). Fig. 2 shows Hill coefficients and logarithms of binding capacities for estimates with a significance of $P \leq 1\%$. Average cluster values are indicated. For most of the binding parameters, the non-linear regression fit improved the estimates so obtained by only a small amount: only the Hill coefficient of the high-affinity sites was changed more substantially in our example (cf. Figs. 1 and 2). This, however, is likely to be due to a low accuracy of radioactivity measurements at low d.p.m. values to which this parameter particularly is very sensitive. In such instances, the original estimates representing mean values of several data combinations were considered to be more accurate.

Sorting of the computed K_d values obtained for different Mg^{2+} concentrations, and their alignment to individual binding sites, followed either subjectively, when a spectrum showed clearly separated clusters of K_d values, or by means of a clustering routine. We have used the K -means clustering (Hartigan, 1975) from the BDMP Statistical Software (Dixon, 1983), which allowed us to define a desired number of clusters (N) to which individual sequences are ordered according to the similarity of their $\log K_d$, $\log B$ and h values. Computations were performed for $N = 3$ to $N = 6$ in each case.

Differences in $\log K_d$ values between individual clusters were tested by Student's t -test (combined with the F test in order to select the proper testing relationship), and non-significantly differing clusters were pooled. [Note that the generalized T^2 test according to Hotelling (1931) would be a more complex, but, in cases of large differences in B and h values between individual clusters, a more appropriate test procedure.] For all Mg^{2+} concentrations employed (0, 0.2, 0.5, 1, 2.75, 4.25 and 10 mM), the final number of significant clusters was always 2 or 3.

Effect of Mg^{2+} on binding parameters

It has to be assumed that Mg^{2+} may substantially change the parameters of a binding site. When the Mg^{2+} concentration

Fig. 1. Evaluation of the binding isotherm: modified affinity spectra method

Panel (b) shows the affinity spectrum obtained for a binding experiment at a relative binding optimum with regard to Mg^{2+} concentration (4.25 mM-Mg^{2+}); the experimental points are plotted double logarithmically in (a). The abscissa shows $\log K_d$ computed for individual partial sequences of the experimental data; the ordinate shows its probability P (in %, plotted logarithmically), estimated by means of the coefficient of linear correlation for this particular sequence. The diagram indicates the existence of three clusters with $P \leq 1\%$. Resolution of the medium-affinity binding sites, of K_d between 10^{-7} and 10^{-6} mol/l , into two clusters, as may seem to follow from the profiles, could not be proven statistically. The cluster with $K_d > 10^{-5} \text{ mol/l}$ is rather broad, with only a few significant lines; it may, at least in part, represent a non-saturable component of the overall binding. (a) Binding isotherm (double logarithmic plot) for three binding sites fitted to the experimental points by means of a non-linear regression routine. Weighted arithmetic means of parameters in individual clusters given in Fig. 2 were used as initial estimates. Triangles mark the position of $\log K_d$ for the particular cluster. Site 1: $K_d = 4.01 \times 10^{-9} \text{ mol/l}$, $B = 2.17 \times 10^{-11} \text{ mol/l}$, $h = 3.18$; site 2: $K_d = 2.88 \times 10^{-7} \text{ mol/l}$, $B = 1.39 \times 10^{-9} \text{ mol/l}$, $h = 1.00$; site 3: $K_d = 6.51 \times 10^{-5} \text{ mol/l}$, $B = 3.30 \times 10^{-7} \text{ mol/l}$, $h = 1.34$. Note that the values of B are given here in terms of maximum values of c_b for oxytocin (mol/l) under the given experimental conditions (not in pmol/mg of protein, as in Figs. 4 and 5).

changes, the assignment of identical binding sites is not always unambiguous. We have therefore carried out a re-clustering of $\log K_d$, $\log B$ and h mean values identified in individual experiments using the K -means routine described above, thus determining the sites of closest similarity within data sets for different Mg^{2+} concentrations; three such groups could be identified (Fig. 3). The effects of Mg^{2+} upon binding parameters were then investigated within these groups.

The results are summarized in Fig. 4. For the high-affinity site with a K_d of about 10^{-9} mol/l , the affinity increased with increasing Mg^{2+} concentration up to approx. 3 mM . Binding to this site was not measurable in the absence of Mg^{2+} ; the decrease in the K_d in the concentration range 0.2 – 2.75 mM-Mg^{2+} was quite dramatic (a factor of 16). A further increase in Mg^{2+} concentration up to 10 mM resulted in slightly suppressed oxytocin binding in our experiments: the K_d increased between 2.75 and 10 mM-Mg^{2+} by a factor of 2.5. The difference between $\log K_d$ values for 2.75 and 10 mM-Mg^{2+} was, however, not statistically significant (Student t test for non-equal variances).

Fig. 2. Plots of binding capacity (a) and Hill coefficients (b; co-operativity plot, cf. Rutschmann *et al.*, 1989) resulting from the affinity spectra analysis of the data in Fig. 1

The graphs comprise estimates of $P \leq 1\%$. Weighted arithmetic means for individual clusters are indicated in the panels with broken lines; open triangles indicate estimated means of $\log K_d$ (corresponding K_d values are given). These values were used as initial estimates for the fit of binding of isotherm (cf. Fig. 1).

Fig. 3. Binding sites of closest similarity obtained in individual binding experiments at variable Mg^{2+} concentrations

A K -means clustering routine (see the text), based on the parameters $\log K_d$, $\log B$ and h , was employed. Three clusters (different symbols) were found to be significantly different in terms of their average distances. The scatter plot shown on the graph is an output of the corresponding BMDP routine (Dixon, 1983). It demonstrates the alignment of sites found in individual experiments, by means of plotting the first two principal components computed from the clustered variables. For computational details, see Hartigan (1975) and Dixon (1983). It can be recognized that individual sites are distinctly different in their binding parameters.

Thus one can conclude that the K_d of this site remains roughly constant above 3 mM-Mg^{2+} . Changes in its binding capacity were quite different: a clear-cut maximum was observed at 1 mM-Mg^{2+} , followed by a steep decrease up to 2.75 mM-Mg^{2+} . Increased Mg^{2+} concentrations did not cause any further change. Fig. 4(c) shows that the Mg^{2+} effect on the Hill coefficient is substantial.

The medium-affinity site (K_d of about 10^{-7} mol/l) was affected by Mg^{2+} in a similar way, except that the increase in K_d in the low Mg^{2+} range was less steep and the decrease above 3 mM was

Fig. 4. Effect of Mg^{2+} on parameters of the binding isotherm (eqn. 2)

Curves 1, 2 and 3 represent high-, medium- and low-affinity sites respectively. Solid lines are arithmetic means of all available estimates (1–7 in number, cf. text). Shaded areas around the lines denote limits of standard errors of means.

Fig. 5. Mg^{2+} -dependence of the B/K_d quotient (logarithm) for individual sites

See the legend to Fig. 4. Values of $\log B/K_d$ are relative to those at 1 mM- Mg^{2+} .

clearly pronounced. Changes in B values had a rather random character, although their trend was similar to that of the high-affinity site.

The K_d of the low-affinity site (around 10^{-5} mol/l) decreased with increasing Mg^{2+} concentration, and B behaved somewhat irregularly. However, the saturability of this site could not be proven and the spectral analysis frequently indicated the presence of several sites, indistinguishable by the statistical tests mentioned above. These sites could not be identified at the highest Mg^{2+} concentration employed in our experiments (10 mM).

DISCUSSION

Our results with sheep myometrial cells indicate that the Mg^{2+} effect, at 1.2 mM- Ca^{2+} , can be observed at the level of both affinity and capacity, and that it is not directly proportional to the Mg^{2+} concentration. The K_d of the high-affinity site at 3 mM- Mg^{2+} was decreased 16-fold compared with that at 0.2 mM- Mg^{2+} . In contrast, the binding capacity was decreased under the same conditions by about 50%; its maximal value, about 4.5-fold

higher, was recorded at 1 mM- Mg^{2+} . Thus a clear-cut optimum of binding capacity and a maximum-binding affinity, at different Mg^{2+} concentrations, were observed in our experiments. The effect of Mg^{2+} on binding can therefore be considered to be rather pronounced. Its absence apparently abolishes binding almost totally; we were not in position to evaluate measurements carried out in Mg^{2+} -free media, since the K_d values of the high- and medium-affinity sites were indistinguishable (Fig. 4a).

The non-monotonic course of Mg^{2+} -dependency was even more clear in the case of the medium-affinity binding site. There was a deep minimum of K_d at 3 mM- Mg^{2+} and a peak in binding capacity at 1 mM- Mg^{2+} . Higher concentrations of Mg^{2+} diminished the binding at this site in all respects. The low-affinity sites displayed a similar tendency with regard to binding capacity; no sites belonging to this cluster could be identified with affinity spectra for 10 mM- Mg^{2+} . However, this cluster is most likely composed of various low-affinity sites which cannot be individually studied with the present experimental and computational approaches.

K_d and B parameters alone cannot describe the effective

binding on single sites. Rather, a conceivable descriptor of this property would be the relative binding, i.e., the ratio of c_b to total ligand concentration ($c_t + c_b$). This ratio is, of course, dependent on the total ligand concentration; for eqn. (2), it runs:

$$c_b/(c_t + c_b) = Bc_t^{h-1}/(Bc_t^{h-1} + K_d^h + c_t^h). \quad (5)$$

For $c_t = K_d$, eqn. (5) becomes:

$$c_b/(c_t + c_b) = B/(B + 2K_d). \quad (6)$$

In most instances, when comparing B and K_d in equal concentration units, $B \ll K_d$. Then the ratio of the two binding parameters, B/K_d , reflects the relative binding at the half-maximal saturation of the site, independent of the power coefficient h . Fig. 5 shows the dependence of this ratio on the Mg^{2+} concentration. This calculated parameter is also dependent on the Mg^{2+} concentration in a non-monotonic manner. Its maximal value for both high- and medium-affinity populations appeared to be at approx. 1 mM- Mg^{2+} . The binding was inhibited quite considerably at concentrations higher than 4 mM- Mg^{2+} , and at very low Mg^{2+} concentrations. This finding contrasts with that of Soloff & Swartz (1976), who describe a potentiation of the oxytocin binding to pig uterus membranes over a broad Mg^{2+} concentration range (up to 10 mM). Reasons for this discrepancy may be several-fold, e.g. species differences, use of different preparations (cells versus a membrane fraction), or simply the fact that the conditions were not fully comparable, since the cited authors performed their measurements at just a single ligand concentration. Thus the potentiating effect of Mg^{2+} on binding of oxytocin to intact sheep myometrial cells appears to occur within a rather narrow Mg^{2+} concentration range; above this range, Mg^{2+} depresses all binding parameters of potential receptors.

The Hill coefficient reflects manifold properties of the ligand-macromolecule interaction, the most obvious of which are for $h > 1$ the positive co-operativity, and for $h < 1$ the negative co-operativity, or the 'quasi-continuous' heterogeneity of the binding energy (Karush & Sonnenberg, 1949; Sips, 1948, 1950; Pliška, 1975). Fig. 4(c) indicates that Mg^{2+} may exercise a regular effect on positive co-operativity for oxytocin binding to high- and medium-affinity sites. The positive co-operativity is dominant here (Hill coefficients attain values of between 1.5 and 2.5) and is dramatically increased at Mg^{2+} concentrations above 2 mM. We believe that this may be another possible explanation for the monotonic course of oxytocin binding found by Soloff & Swartz (1976) in the sow; when using a single concentration of the ligand, as was the case in the cited study, the effect of co-operativity cannot be recognized. Experiments of that kind are therefore of little value for the analysis of binding mechanisms. Positive co-operativity on the medium-affinity binding site is considerably weaker (maximal Hill coefficient 1.5) and the effect of Mg^{2+} is less pronounced (Fig. 4). Nevertheless, there is a clear-cut minimum of h at about 1 mM- Mg^{2+} , in marked contrast to the affinity, which has its maximum at this concentration. The two sites are therefore clearly different with regard to the effect of Mg^{2+} . As for the low-affinity site(s), no systematic effect of Mg^{2+} can be recognized (Fig. 4); positive co-operativity is not pronounced (h is maximally 1.25).

Our analysis employs a slightly unconventional evaluation method, the K -means clustering, and it is not amiss to add a few comments on its use. It may turn out to be generally difficult to perform any studies in a multiple binding sites system, since an unambiguous assignment of an experimental finding to a particular binding site cannot be done in a completely objective

manner. To return to our case, due to a shift caused by Mg^{2+} the binding parameters might be changed to such an extent that comparison between two Mg^{2+} concentrations (in general, two sets of conditions) may become very problematic, especially when one of the populations could not be measured. Clustering routines may help to solve the problem only incompletely, since they are not statistical procedures and do not allow a determination of the error probabilities. However, we found them useful for a preliminary grouping of binding site populations with the 'most similar' binding parameters. Differences between classes so defined can, in the next step, be tested for their significance using common statistical routines.

Until now, no dose-response studies with oxytocin on sheep uterus have been reported; a comparison of Mg^{2+} effects upon binding and response is therefore lacking. However, binding parameters with sheep cells (K_d and B) for both high- and medium-affinity sites, and the pD_2 value for the rat uterus display clear-cut similarities in the biphasic nature of the Mg^{2+} dependence (Krejčí *et al.*, 1964); it can be tentatively concluded that at least a part of the Mg^{2+} effect upon the uterotonic response to oxytocin is exercised at the receptor level. This is in agreement with the conclusions reached by Antoni & Chadio (1989).

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REFERENCES

- Antoni, F. A. & Chadio, S. E. (1989) *Biochem. J.* **257**, 611-614
- Atkins, G. L. (1973) *Eur. J. Biochem.* **33**, 175-180
- Ayad, V. J. & Wathes, D. C. (1989) *J. Endocrinol.* **123**, 11-18
- Bentley, P. J. (1965) *J. Endocrinol.* **32**, 215-222
- Crankshaw, D., Gaspar, V. & Pliška, V. (1990) *J. Receptor Res.* **10**, 269-285
- Debus, E., Weber, K. & Osborn, M. (1983) *EMBO J.* **2**, 2305-2312
- Dixon, W. J. (ed.) (1983) *BMDP Statistical Software*, University of California Press, Berkeley, CA
- Hartigan, J. A. (1975) *Clustering Algorithms*, Wiley, New York
- Hotelling, H. (1931) *Ann. Math. Stat.* **2**, 360-378
- Karush, F. & Sonnenberg, M. (1949) *J. Am. Chem. Soc.* **71**, 1369-1376
- Krejčí, I. & Poláček, I. (1968) *Eur. J. Pharmacol.* **2**, 393-398
- Krejčí, Poláček, I., Kupková, B. & Rudinger, J. (1964) in *Oxytocin, Vasopressin and their Structural Analogues* (Rudinger, J., ed.), pp. 117-123, Pergamon Press, Oxford
- Lopez Bernal, A., Phipps, S. L., Rosevear, S. K. & Turnbull, A. C. (1989) *Br. J. Obstet. Gynaecol.* **96**, 1108-1110.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Munsick, R. A. (1960) *Endocrinology* (Baltimore) **66**, 451-457
- Munsick, R. A. & Jeronimus, S. C. (1965) *Endocrinology* (Baltimore) **76**, 90-96
- Pliška, V. (1975) *Ann. N.Y. Acad. Sci.* **248**, 480-493
- Pliška, V. (1988) *J. Receptor Res.* **8**, 245-259
- Pliška, V., Heiniger, J., Müller-Lhotsky, A., Pliska, P. & Ekberg, B. (1986) *J. Biol. Chem.* **261**, 16984-16989
- Rutschmann, M. A., Heiniger, J., Pliška, V. & Solms, J. (1989) *Lebensm. Wiss. Technol.* **22**, 240-244
- Sips, R. (1948) *J. Chem. Phys.* **16**, 490-495
- Sips, R. (1950) *J. Chem. Phys.* **18**, 1024-1026
- Soloff, M. S. & Swartz, T. L. (1976) *J. Biol. Chem.* **249**, 1376-1381
- Soloff, M. S. & Grzonka, Z. (1986) *Endocrinology* (Baltimore) **119**, 1564-1569
- Tobler, H. J. & Engel, G. (1983) *Naunyn-Schmiedeberg's Arch. Pharmacol.* **322**, 183-192