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ROLE OF CALCIUM AND PROSTAGLANDINS IN THE ANTIDIURETIC HORMONE RESPONSE

EFFECT OF IONOPHORE A23187

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Abstract—The calcium ionophore A23187 (IP) inhibited the antidiuretic hormone (ADH)-stimulated hydro-osmotic response in toad urinary bladder but had no effect on the osmotic transfer of water in the absence of hormone. Extracellular calcium was necessary for this effect at lower but not at higher IP concentrations. The hydro-osmotic response to exogenous cyclic AMP was unaltered by IP, but the same response produced by inhibition of phosphodiesterase was reduced significantly. Cyclic AMP concentrations in isolated toad bladder epithelial cells were reduced by 50% with IP or exogenous prostaglandin E_2 (PGE_2). Indomethacin, a prostaglandin synthesis inhibitor, prevented the inhibitory actions of the IP on the ADH-mediated response. Collectively, these observations suggest a key role for cellular calcium in modulating the actions of antidiuretic hormone and are consistent with the hypothesis that the ionophore, through increasing intracellular calcium, stimulates the synthesis of prostaglandins which have a negative feedback on adenylcyclase. This effect would terminate the action of the hormone.

The biochemical steps leading to the action of antidiuretic hormone (ADH) are not fully understood but are believed to involve a series of complex steps that include ADH binding to its membrane receptor [1], activation of adenylcyclase, the production of adenosine 3′-5′-cyclic monophosphate (cyclic AMP) [2], and the activation of protein kinase [3] followed by an increase in membrane permeability and an increase in osmotic water flow. Recent observations have also implicated a role for microtubules and microfilaments in ADH actions [4].

The ADH response may be modified in part by a change in cellular calcium metabolism. The following observations are consistent with this hypothesis. In man, chronic hypercalcemia can impair the maximal urine-concentrating ability [5], and acute hypercalcemia diminishes the renal responsiveness to exogenous ADH. In isolated epithelia, elevated extracellular calcium inhibits the ADH and theophylline mediated hydro-osmotic response but not that induced by exogenous cyclic AMP [6–8]. Low calcium concentrations, however, augment the ADH hydro-osmotic response [6].

Recently, calcium ionophores have been reported to inhibit as well as enhance the *in vitro* hydro-osmotic response to ADH [9, 10]. A preliminary report has suggested that the inhibitory effects of the calcium ionophore are mediated by endogenous prostaglandins (PG) [11]. Prostaglandins inhibit the hormone induced increase in osmotic water flow and, like calcium, do not modulate exogenous cyclic AMP-mediated effects [12, 13]. Recently, Berl [14] has suggested that prostaglandins attenuate the ADH response by primarily interfering with membrane calcium transport. The present study further probes the relationships among calcium, prostaglandins, and the action of antidiuretic hormone in an isolated epithelium, the toad urinary bladder, utilizing the calcium ionophore A23187.

METHODS

Toads, *Bufo marinus*, were obtained from a commercial supplier (NASCO, Fort Atkinson, WI) and kept in terraria at 25°.

Toad urinary bladder preparation. Toad urinary bladder "sacs" were set up and incubated in Ringer's solution as described previously [15]. For the measurement of the hydro-osmotic response, bladder sacs were filled with 4 ml of 1/10 Ringer's solution and immersed so that the serosal (blood) side was bathed in 30 ml of Ringer's solution. The size of the bladder sacs was kept constant so as to minimize variations in surface area.

Osmotic water flow was measured gravimetrically by weighing the bladder sacs before and after a 30-min or 60-min period of incubation in the presence of an osmotic gradient [15]. The experimental design is described where appropriate in the text and tables. The Ringer's solution was constituted as follows (millimolar concentrations): NaCl, 111; KCl, 3.35; $CaCl_2$, 2.7; $MgCl_2$, 0.5; $NaHCO_3$, 4.0; and glucose, 5. This solution was aerated throughout, and the pH was 8.0 at the start of the experiment and did not fall below 7.8 by the end of the incubation period.

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Table 1. Effect of Ca^{2+} ionophore (IP) on the hydro-osmotic response to vasopressin (ADH, 10 mU/ml)*

		Water movement (mg/30 min)		
		Period I (vasopressin)	Period II (vasopressin + IP in A)	P for difference† (A vs B Period II)
(A)	10^{-6} M IP in serosal solution in Period II (6)	867 ± 19	567 ± 44	<0.01
(B)	Control (6)	859 ± 58	887 ± 72	
(A)	5×5^{-5} M IP in serosal solution in Period II (12)	1225 ± 58	552 ± 35	<0.001
(B)	Control (12)	1182 ± 89	1013 ± 58	

* Paired hemi-bladders were incubated in Ringer's solution. Values are means ± S.E. The number of experiments is indicated in parentheses.

† Statistical analysis of a paired sample *t*-test.

‡ A 15-min preincubation with IP preceded period II. The control bladder lobe received an equivalent amount of ionophore solvent DMSO. Period I and Period II were separated by a 30-min washout interval.

In some instances the calcium concentration was reduced to 0.027 mM or calcium was completely omitted from the solution. The osmolality was kept constant by the addition of sucrose.

Cyclic AMP determination. Toad bladders were mounted as sacs, filled with 10 ml of calcium-free Ringer's solution, and aerated at room temperature for 45 min. The sacs were gently massaged, and the mucosal solutions containing the released epithelial cells were collected and centrifuged at 3000 *g* for 20 min. The cells were resuspended in 2 ml of calcium-rich Ringer's solution and aerated for 30 min at room temperature. The epithelial cells were isolated from paired bladder sacs and subjected to treatments as indicated in the text. Following the treatment period, the cells were resuspended in PBS-gel (0.1 M phosphate buffered saline, 0.1% gelatin, pH 7.0), homogenized in a Polytron, and placed in a 100° water bath for 10 min. The samples were then centrifuged at 5000 *g* for 15 min, and aliquots of the supernatant fraction were removed for cyclic AMP determinations. The pellet was washed and resuspended in 0.1 N NaOH, and protein concentrations were determined by the dye-binding assay of Bradford [16].

Cyclic AMP was determined in triplicate by radioimmunoassay using 0.1 ml aliquots of sample [17]. The antiserum employed was obtained from rabbits immunized with succinyl cyclic AMP bovine serum albumin. The antiserum was diluted with 1:400 normal rabbit serum (in 0.01 M phosphate buffered saline) and used at a final dilution of 1:200,000. The labeled ligand, succinyl cyclic AMP tyrosine methylester (Sigma Chemical Co., St. Louis, MO), was radioiodinated with ^{125}I (New England Nuclear Corp., Boston, MA) by the Chloramine T procedure and separated by gel filtration on Sephadex G-25. All results are expressed as picomoles of cyclic AMP per milligram protein.

Drugs. The following drugs and hormones were used: indomethacin, 8-arginine vasopressin and cyclic adenosine monophosphate (Sigma Chemical Co.). The calcium ionophore A23187 was a gift from Robert Hosley of the Lilly Research Laboratories.

Table 2. Effects of Ca^{2+} ionophore (IP) on the hydro-osmotic response to vasopressin in low Ca Ringer (0.027 mM*)

		Water movement (mg/30 min)		
		Control bladder lobe (no ionophore)	Experimental lobe (ionophore present)	P for difference†
(A)	ADH (10 mU/ml)			
	10^{-6} M IP‡ (6)	809 ± 51	731 ± 41	NS
	5×10^{-5} M IP (6)	1102 ± 110	438 ± 56	<0.01
(B)	Ca-free Ringer			
	5×10^{-5} M IP (12)	1159 ± 61	656 ± 38	<0.01

* Paired hemi-bladders were incubated in a Ringer's solution containing 0.027 mM calcium or in calcium-free solutions (B). Bladder sacs were allowed to equilibrate in Ringer's solution for 30 min and then were rinsed in low calcium or calcium-free medium, mounted, and immediately used in the measurement of osmotic water flow. The bladders were exposed to the low calcium environment for no longer than 45 min. Values are means ± S.E. and the number of experiments is indicated in parentheses.

† Statistical analysis was performed using a paired sample *t*-test. NS = not statistically significant ($P > 0.05$).

‡ The ionophore was added to the experimental lobe for a 15-min preincubation before the measurement of osmotic water flow. The IP vehicle was added to the control lobe.

Table 3. Effects of Ca^{2+} ionophore (IP) on the hydro-osmotic responses of the toad bladder to cyclic AMP, dibutyryl cyclic AMP and 3-isobutyl-1-methylxanthine (MIX)*

		Water movement (mg/60 min)		
		Control bladder lobe (no ionophore)	Experimental lobe (ionophore present)	P for difference
(A)	Cyclic AMP (2 mM)			
	10^{-6} M IP† (6)	714 ± 144	503 ± 102	NS
	5×10^{-5} M IP (6)	690 ± 140	559 ± 132	NS
(B)	Dibutyryl cyclic AMP (1 mM)			
	10^{-6} M IP (10)	1600 ± 77	1268 ± 89	<0.01
	5×10^{-5} M IP (6)	1241 ± 329	400 ± 82	<0.05
(C)	MIX (0.5 mM)			
	10^{-6} M IP (10)	921 ± 97	410 ± 92	<0.01
(D)	MIX (0.5 mM) + cAMP (2 mM)			
	5×10^{-5} M IP (10)	1390 ± 140	800 ± 40	<0.01

* Paired hemi-bladders were incubated in Ringer's solution. Values are means ± S.E. The number of experiments is indicated in parentheses.

† The ionophore was added to the experimental lobe 15 min prior to the measurement of osmotic water flow.

The ionophore was dissolved in a stock solution of dimethyl sulfoxide (DMSO) to a concentration of 0.05 M. PGE_2 was a gift from Dr. John E. Pike of the Upjohn Co.

The statistical analysis was performed using Student's *t*-test for differences between two sample means [18].

RESULTS

Effects of Ca^{2+} ionophore A23187 on the hydro-osmotic response to ADH (10 mU/ml). When 10^{-6} M calcium ionophore A23187 (IP) was added to the serosal side of the toad urinary bladder, the ADH response was reduced by approximately 40% (Table 1). This inhibition was enhanced at higher ionophore concentrations, whereas 10^{-7} M IP had no effect on the hormone-mediated response (not shown). There was no detectable change in the rate of water movement due to an osmotic gradient across the toad bladder in the presence of ionophore alone, even at a concentration of 5×10^{-5} M.

To determine if external calcium was necessary for the inhibitory effects of the IP, toad bladder sacs were incubated in low calcium medium (0.027 mM). The concentration of calcium in the medium was confirmed using atomic absorption spectrophotometry. Lower calcium concentrations had little effect on the ADH-mediated response as judged by the responses in Table 1 compared to the control bladder lobe in Table 2. When the external serosal calcium concentration in the Ringer's solution was reduced, the previously observed inhibitory effect of 10^{-6} M ionophore on the ADH-mediated hydro-osmotic response was not detected (Table 2). However, at higher ionophore concentrations a significant inhibition of the ADH response remained, even in the absence of added external calcium (Table 2). These results suggest that at low IP concentations external calcium is necessary to produce an inhibitory effect, whereas with increased IP concentrations external calcium is no longer essential in eliciting a response.

Effects of ionophore on cAMP-induced osmotic water flow. The effect of vasopressin on the amphibian bladder involves a complex series of changes, and the effects of ionophore may occur at one or more of these sites.

The hydro-osmotic response to exogenous cyclic AMP was unaffected by the ionophore at the concentrations tested (Table 3). Interestingly, the IP inhibited the actions of the exogenously administered cyclic AMP analog, dibutyryl cyclic AMP (db-cAMP) (Table 3). db-cAMP is the more lipid soluble analog of cyclic AMP and thus easily crosses cellular membranes. However, in addition to the dibutyryl's cyclic AMP-like effects, it has been suggested that db-cAMP may also inhibit cAMP phosphodiesterase [19]. Such an effect would elevate endogenous cAMP concentrations in addition to its direct cellular actions of the added nucleotide. Certainly the greater water flow response observed with the analog is consistent with these dual actions (Table 3).

If the ionophore acts on the adenylcyclase system, it is not surprising to observe an inhibition of the db-cAMP response if this analog also acts by elevating endogenous cyclic AMP concentrations. There is additional support for this concept, in that the ionophore inhibited the hydro-osmotic response seen in the presence of 3-isobutyl-1-methylxanthine (MIX), a phosphodiesterase inhibitor (Table 3).

To determine if the ionophore inhibition of the db-cAMP response is a result of the db-cAMP effects on phosphodiesterase, an experiment was designed to mimic the two actions of db-cAMP. In the presence of MIX plus added cyclic AMP, the IP produced a similar decrease in the water response as compared to MIX alone (Table 3).

Effects of ionophore in the presence of prostaglandin synthesis inhibitors. The role of prostaglandins as intracellular modulators has gained wide acceptance. Administered prostaglandins inhibit the hormone-induced increase in osmotic water flow and like calcium, or as reported here calcium ionophore, do not modulate the effects of exogenous cyclic AMP [12, 13].

The inhibitory effects of the IP may be mediated

Table 4. Effects of Ca^{2+} ionophore (IP) on the hydro-osmotic response to vasopressin (ADH, 10 mU/ml) or methylxanthine (MIX) (0.5 mM) in the presence of 10^{-5} M indomethacin or 10^{-5} M mepacrine*

		Control bladder lobe (no ionophore)	Experimental lobe (ionophore present)	P for difference
			Water movement (mg/30 min)	
(A)	ADH + indomethacin			
	10^{-6} M IP (8)	1180 ± 64	945 ± 74	NS
	5×10^{-5} M IP (8)	1136 ± 80	758 ± 45	<0.01
			Water movement (mg/60 min)	
(B)	MIX + indomethacin			
	10^{-6} M IP (6)	1447 ± 120	1276 ± 92	NS
(C)	ADH + mepacrine			
	10^{-6} M IP (9)	932 ± 48	821 ± 25	NS

* Paired hemi-bladder sacs were incubated in Ringer's solution. Indomethacin or mepacrine was added 30 min before the addition of ADH. The ionophore was added to the experimental lobe 15 min prior to the measurement of osmotic water flow. The results are expressed as means ± S.E. The number of experiments is indicated in parentheses.

through the endogenous production of prostaglandins. In the presence of the prostaglandin synthesis inhibitor, indomethacin, the IP at 10^{-6} M had no effect on the ADH-induced increase in osmotic water movement (Table 4). However, at higher ionophore concentrations, indomethacin did not protect against IP inhibitory effects. Indomethacin also protected against the inhibition by IP (10^{-6} M) of the methylxanthine response. Mepacrine, a phospholipase inhibitor [20], also prevented the inhibitory actions of IP (Table 4). These results suggest that, at low ionophore concentrations, inhibition of the antidiuretic hormone response may be due to IP stimulation of phospholipase activity and an increase in prostaglandin synthesis.

Effects of calcium ionophore and prostaglandins on cellular cyclic AMP concentrations. If the ionophore inhibits adenylcyclase, the cyclic AMP concentrations within the cells should decrease. In addition, if the ionophore acts by stimulating the release of endogenous prostaglandins, then the addition of exogenous prostaglandins should also reduce cellular nucleotide concentrations.

Cyclic AMP was measured in epithelial cells isolated from toad urinary bladders. The cells were incubated with and without hormone and in the presence of the IP or PGE_2. All cells were preincubated with methylxanthine, a phosphodiesterase inhibitor, to prevent breakdown of cyclic AMP during isolation procedures. The calcium ionophore significantly reduced the levels of cAMP in the antidiuretic hormone stimulated or unstimulated cells (Table 5). Interestingly, PGE_2 produced dose-dependent effects. At low doses (10^{-8} M), PGE_2 inhibited the formation of cyclic AMP, whereas at relatively high concentrations (10^{-5} M) PGE_2 stimulated cyclic AMP formation. Such dose-dependent effects of prostaglandins on cyclic AMP accumula-

Table 5. Effects of calcium ionophore and PGE_2 on cyclic AMP levels in isolated toad bladder epithelial cells*

	cAMP (pmoles/mg protein)	P for difference
Stimulated		
ADH (10 mU/ml) (8)	54 ± 6.1	<0.01
ADH + 5×10^{-5} M IP (8)	26 ± 5.8	
ADH (10 mU/ml) (8)	51 ± 6.4	<0.05
ADH + 10^{-6} M IP (8)	31 ± 4.9	
ADH (10 mU/ml) (10)	47 ± 4.9	<0.05
ADH + 10^{-5} M PGE_2 (10)	66 ± 3.2	
ADH (10 mU/ml) (8)	44 ± 5.4	<0.05
ADH + 10^{-8} M PGE_2 (8)	23 ± 3.7	
Unstimulated		
Control (6)	27 ± 3.1	<0.05
5×10^{-5} M IP (6)	13 ± 3.0	
Control (6)	24 ± 4.1	<0.05
10^{-5} M PGE_2 (6)	55 ± 3.2	
10^{-8} M PGE_2 (6)	19 ± 2.7	

* Toad bladder epithelial cells were isolated as described in Methods. The epithelial cells were incubated with ionophore 15 min prior to the addition of ADH. After an additional 15 min of incubation the cells were suspended in PBS gel, homogenized, and placed in a 100° water bath. Cyclic AMP was measured as described in Methods. Results are expressed as means ± S.E. The number of experiments is indicated in parentheses.

tion have been observed previously [21]. Indomethacin, a prostaglandin synthesis inhibitor, reversed the inhibitory effects of the ionophore on cyclic AMP accumulation. The ADH-stimulated cells had 63 ± 3 pmoles cAMP/mg protein in the absence of ionophore and 58 ± 3 pmoles cAMP/mg protein in the presence of 10^{-5} M indomethacin (added 15 min prior to IP administration) and 10^{-6} M IP (added 15 min prior to ADH administration) (not statistically significant using Student's *t*-test for six observations).

DISCUSSION

Calcium is considered to be one of the key factors in the regulation of a variety of general cellular processes including the regulation of sodium and water transport in epithelia. Calcium has been viewed as an intracellular second messenger that triggers a series of cellular events as part of hormonal responses. Alterations in the calcium ion concentration have been shown to influence the hydro-osmotic effects of ADH [6–8].

The hydro-osmotic response of the toad urinary bladder to antidiuretic hormone was inhibited by the calcium ionophore, whereas the osmotic transfer of water in the absence of ADH was unaffected. The effect of the IP on the ADH hydro-osmotic response required calcium at low ionophore concentrations, but not at higher ionophore concentrations. The differences could have been due to a dose-dependent ability of IP to mobilize intracellular calcium at higher concentrations or perhaps we were seeing potentially toxic uncoupling of mitochondrial oxidative phosphorylation by A23187 [22].

Cyclic AMP, as well as phosphodiesterase inhibitors, mimics the action of antidiuretic hormone when added exogenously. The hydro-osmotic response to exogenous cyclic AMP was unaffected by ionophore, while that due to 3-isobutyl-1-methylxanthine and dibutyryl cyclic AMP was considerably reduced. The effect of dibutyryl cAMP is probably not solely exerted by mimicking endogenous nucleotide but also through inhibition of phosphodiesterase. Such a dual action would help explain the large discrepancy between the abilities of the IP to block the db-cAMP and not the exogenous cyclic AMP response. This concept is supported by the action of the ionophore to inhibit the methylxanthine-mediated hydro-osmotic response. This clear inhibition of the db-cAMP hydro-osmotic response suggests that the indirect effect of db-cAMP on the accumulation of endogenous cyclic AMP may be its predominant action.

These results suggest that elevation of intracellular calcium by ionophore and inhibition of the ADH-mediated response occur at a site prior to cyclic AMP formation, perhaps on hormone-sensitive adenylcyclase.

The ADH-induced increase in cyclic AMP has been suggested to cause an increase in intracellular calcium [23]. For instance, ADH increases calcium efflux from toad urinary bladder previously labeled with $^{45}Ca^{2+}$ [24, 25], and these changes in intracellular calcium may function to regulate the action of the hormone. Pietras *et al.* [26] have evidence which suggests that ADH increases total cellular calcium in isolated frog bladder epithelial cells, and Cuthbert and Wong [24] have shown an increased calcium exchange in response to ADH or cyclic AMP. Since these elevations in intracellular calcium during hormonal stimulation are apparently inhibitory to the actions of ADH, it is reasonable to propose that calcium functions as a negative feedback regulator.

Interestingly, exogenously administered prostaglandins mimic the actions of the calcium ionophore to inhibit the ADH-mediated response. Prostaglandins inhibit the hormone-induced increase in osmotic water flow and like the ionophore, or calcium, do not alter the permeability changes seen with exogenous cyclic AMP [12, 13].

In the presence of indomethacin, an inhibitor of prostaglandin biosynthesis, the ionophore (10^{-6} M) had no effect on the ADH or methylxanthine-induced increase in osmotic water movement. These observations suggested that the ionophore, by elevation of intracellular calcium, stimulated the prostaglandin biosynthesis cascade, probably through activation of a phospholipase. Mepacrine, a phospholipase inhibitor, also prevented the inhibitory effects of the ionophore on the ADH response, substantiating the suggestion that the ionophore was probably activating an endogenous phospholipase. Stimulation of prostaglandin biosynthesis by ionophore A23187 has also been observed in rat inner medullary slices [27].

At higher ionophore concentrations indomethacin failed to protect against the inhibitory effects of the ionophore. One possible explanation for this apparent inconsistency is that at higher concentrations we saw a direct, perhaps toxic effect of the ionophore independent of calcium. Alternatively, at these concentrations the ionophore may cause a greater stimulation of phospholipase resulting in increased release of arachidonic acid and synthesis of PGE which is not blocked by this concentration of indomethacin. Another possible explanation is that very high calcium concentrations resulting from high IP concentrations have direct effects on adenylcyclase, and indeed Hynie and Sharp [28] have shown that the adenylcyclase isolated from toad urinary bladder is inhibited by calcium.

If the action of the ionophore reflects a decrease in adenylcyclase activity, cyclic AMP concentrations should be lower. The ionophore prevented the increase in cyclic AMP production induced by hormone and significantly reduced the concentration of cyclic AMP in isolated toad bladder epithelial cells in the absence of hormone. A similar effect of A23187 on cyclic AMP formation has been observed by Wiesmann *et al.* [29] on scraped toad bladder epithelial cells. The effect of ionophore on cyclic AMP concentrations was prevented by pretreatment with indomethacin, further suggesting that the inhibitory effects of the ionophore are mediated through endogenous prostaglandins.

The effects of prostaglandins on cyclic AMP concentrations in isolated toad bladder epithelial cells are dose-dependent in that, at 10^{-8} M, PGE_2 inhibited formation but at higher concentrations (10^{-5} M) it stimulated adenylcyclase. Such a dose–response relation may help to explain the variety of

cyclic AMP changes that have been reported in the literature. Omachi *et al.* [21] reported similar dose-dependent effects of prostaglandins in toad bladder epithelium. Grantham and Orloff [13] were the first to suggest that prostaglandins formed within ADH-sensitive cells would serve to attenuate hormonal effects and modulate the water permeability response. Recently, it has been suggested that prostaglandins may alter membrane calcium transport and that this may explain prostaglandin inhibitory effects on ADH [14]. Such an hypothesis is consistent with our observations as changes in cellular calcium could depress adenylcyclase activity.

The report by Hardy [10] that ionophore A23187 increases basal water flow in frog urinary bladder comparable to that of ADH appears contrary to the present results. However, in that study bladders were pretreated with ionophore in the absence of external calcium and exposed subsequently to increases in calcium concentrations. In the absence of external calcium, the ionophore could have been increasing calcium movements out of the cell to below some critical cellular concentration, and by placing calcium back in the external medium suddenly reversed the calcium flow to trigger a water permeability response.

This effect could mimic the hormonal response as it has been reported that ADH stimulates calcium efflux from toad bladder cells [24]. Hardy [10] also reported, however, that in the presence of external calcium the ionophore blunted the ADH hydro-osmotic response, similarly to what has been observed here, although the mechanism of that inhibition was not reported previously. It is clear, however, that alteration in cellular calcium concentrations can have extensive effects on cellular permeability properties and its responsiveness to hormone.

The mechanism whereby ionophore A23187 alters the hormonal-mediated hydro-osmotic response is consistent with increasing intracellular calcium and subsequent activation of a phospholipase, stimulation of prostaglandin biosynthesis, depression of adenylcyclase activity, and inhibition of hormone-mediated activation. In view of previous reports concerning hormone–calcium interactions, it is reasonable to propose that ADH may through mobilization of intracellular calcium and stimulation of prostaglandin biosynthesis regulate, by feedback, its own response.

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