Physiology/Pharmacology and Neurobiology. Regarding the articles "Chromostatin, a 20-amino acid peptide derived from chromogranin A, inhibits chromaffin cell secretion" by Estelle Galindo, Attila Rill, Marie-France Bader, and Dominique Aunis, and "Chromostatin inhibits catecholamine secretion in adrenal chromaffin cells by activating a protein phosphatase" by Estelle Galindo, Jean Zwiller, Marie-France Bader, and Dominique Aunis, which appeared in number 4, February 15, 1991, and number 16, August 15, 1992, of Proc. Natl. Acad. Sci. USA (88, 1426–1430, and 89, 7398–7402), respectively, the authors request that it be noted that further studies [Daniel T. O’Connor (School of Medicine, University of California, San Diego), personal communication; L. Taupenet and D.A., unpublished work] have shown that the results reported in these papers were due not to the synthetic chromogranin (Neosystem, Strasbourg, France) used but rather to a contaminant. This conclusion is based on the following data. The activity of the Neosystem preparation (purity, 97.22% as judged by the 0–50% acetonitrile profile provided by the manufacturer and confirmed by us) on catecholamine secretion was unaffected by prior treatment with Pronase or H$_2$O$_2$, an observation that weighs against the active component being a peptide. When the HPLC elution was continued to high concentrations of acetonitrile (>65%), a second peak eluted, as revealed from its absorbance at 212–214 nm. Judging from the high acetonitrile concentration required for its elution, this component is very hydrophobic. Its UV spectrum showed maxima at 207.5 nm, 230 nm, and 280 nm. Since there are no tryptophan, tyrosine, or phenylalanine residues in the chromostatin sequence, it seems unlikely that this component is a peptide. Amino acid analysis performed by Neosystem and by us confirmed that the hydrophobic peak is not due to a peptide. NMR spectra showed the presence of aromatic ring(s) and of acetyl and/or isopropyl group(s); it was not possible to identify its complete atomic structure because of the lack of material. Gas chromatography/mass spectrometry revealed a single component with a molecular mass of 455. These structural analyses indicate the presence of a contaminant, perhaps a chemical product or its derivative used during peptide synthesis. We have now repeated the original experiments using the HPLC-purified contaminant and also HPLC-purified chromostatin. The contaminant was found to be quite active in inhibiting nicotine- and high-K$^+$-evoked catecholamine release from bovine chromaffin cells in culture although it did not reproduce the stimulatory effect on serine/threonine phosphatase activities previously observed with the synthetic chromostatin. Electrophysiological experiments on chromaffin cell calcium channels could not be repeated because of the limited quantity of the contaminant. The original observation reported by Simon et al. (1) and subsequently confirmed by D. T. O’Connor and B. M. Gill (personal communication) indicated that peptides derived from chromogranin A are able to control catecholamine secretion in chromaffin cells. The complete nature of the active peptide remains an open question.

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Chromostatin inhibits catecholamine secretion in adrenal chromaffin cells by activating a protein phosphatase

(adrenal medulla/ catecholamines/ exocytosis/ chromogranin)

ESTELLE GALINDO, JEAN ZWILLER, MARIE-FRANCE BADER, AND DOMINIQUE AUNIS*

Institut National de la Santé et de la Recherche Médicale, U-338, Biologie de la Communication Cellulaire, 5, rue Blaise Pascal, 67084 Strasbourg Cedex, France

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ABSTRACT Chromostatin is a 20-residue peptide derived from chromogranin A (CGA), the major soluble component of secretory granules in adrenal medullary chromaffin cells. One known biological function of chromostatin is to inhibit the secretagogue-evoked catecholamine secretion from chromaffin cells. Putative receptors are present on the chromaffin-cell plasma membrane, and the activation of such receptors leads to the inhibition of L-type voltage-sensitive calcium channels. We report here that exposure of chromaffin cells to chromostatin modifies neither cAMP and cGMP levels nor protein kinase C activity but does provoke the activation of soluble protein phosphatase (PPase) type 2A in a dose-dependent manner compatible with the peptide concentration inhibiting catecholamine secretion. The activation of the PPase as well as the inhibition of both secretagogue-induced Ca2+ entry and catecholamine secretion by chromostatin were all blocked by okadaic acid, a specific PPase inhibitor. We suggest that chromostatin directly or indirectly stimulates PPase-2A, dephosphorylating a target protein and lowering its activity in the secretory process.

Chromogranin A (CGA) is a member of a family of acidic secretory proteins, the chromogranins/secretogranins, which are widely distributed in peptidergic endocrine cells and neurons (1). At the subcellular level, CGA is found in the soluble core of hormone and neurotransmitter storage vesicles and is released from them by exocytosis after cell stimulation.

CGA remains the topic of many unanswered questions concerning its possible functions, although recent data suggest a possible prohormonal role in the autocref and/or paracrine modulation of regulated secretion. The sequence of pancreastatin, a 49-residue peptide from porcine pancreas inhibiting insulin secretion from endocrine pancreatic islets (2, 3), amylase release from exocrine pancreas (4), and acid secretion from parietal cells (5), is fully contained within the sequence of porcine CGA. CGA is also the precursor of chromostatin, a peptide that exerts a negative-feedback control on the secretory activity of chromaffin cells (6, 7). In addition, an inhibitory autocref role of CGA on propiomelanocortin secretion (8) and a regulatory role of aminergic CGA-derived peptides on the secretion of calcitonin gene-regulating products (9) and on vascular contractile responses (10) have been reported. Together these observations strongly support the view that CGA is a prohormone.

In a previous report, we described the purification of two CGA-derived peptides active on catecholamine secretion from cultured chromaffin cells (7). A corresponding 20-amino acid peptide named chromostatin was synthesized and found to completely inhibit catecholamine secretion in the nanomolar to micromolar range. Bovine chromaffin cells possess specific receptors for chromostatin the activation of which markedly decreases Ca2+ entry through voltage-gated L-type calcium channels (11). The main purpose of the present study was to investigate how the inhibitory effect of chromostatin is achieved. We suggest that a protein phosphatase (PPase) may be responsible, at least partially, for the chromostatin-induced inhibition of catecholamine release.

MATERIALS AND METHODS

[3H]Norepinephrine (Noradrenaline) Release Assay. Chromaffin cell cultures were prepared as in ref. 12. Four- to six-day-old cultures were used for [3H]norepinephrine secretion, which was done and expressed as described in ref. 12.

Protein Kinase C (PKC) Activity and Cyclic Nucleotide Measurements. PKC activity was determined as in ref. 13. Measurement of cyclic nucleotide levels was done in chromaffin-cell supernatant with assay kits (Amersham), following the manufacturer’s instructions.

45Ca2+-Uptake Experiments. 45Ca2+ (13.5 mCi/mg of Ca; CaCl2 salt; New England Nuclear/DuPont; 1 Ci = 37 GBq) influx into cells was measured as described (14, 15).

Determination of PPase Activity. Histone was phosphorylated with cAMP-dependent protein kinase as described by Zwiller et al. (16), and the 32P-phosphorylated histone was recovered according to the procedure reported by Meisler and Langan (17). Chromaffin cells were grown for 3–6 days at a density of 5 x 106 cells on 35-mm-diameter plastic dishes. After extensive washing, cells were incubated for 10 min in Locke’s solution in the presence or absence of 100 nM of the indicated peptide. Cells were subsequently scraped off in 5 mM Tris-HCl, pH 7.5/0.4 mM EDTA and centrifuged. PPase activity with phosphohistone as substrate was determined in both the supernatant and the membrane fraction extracted with 1% 3-[3-cholamidopropyl]dimethylammonio)-1-propane sulfonate (CHAPS) by measuring the release of 32P. The assay mixture (80-μl total volume) contained 50 mM Tris-HCl, pH 7.5/0.5 mM dithiothreitol/1 mM EDTA/32P-phosphorylated histone (2 μM in phosphate content) and, when indicated, PPase activity was inhibited by adding okadaic acid to the enzyme mixture. Dephosphorylation reactions were routinely done at 30°C for 5 min and stopped by adding 100 μl of 1 M H2SO4/1 mM potassium phosphate. The released radioactive phosphate was extracted as a phosphomolybdate complex and measured according to the method of Killilea et al. (18). To ensure linearity, the dephosphorylation was kept within a limit of 20% conversion from the phosphorylated form.

Abbreviations: CGA, chromogranin A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PPase, protein phosphatase. *To whom reprint requests should be addressed.

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RESULTS

Effect of Chromostatin on Secretagogue-Induced Catecholamine Release from Chromaffin Cells. Previous work from our laboratory has shown that chromostatin produces a dose-dependent inhibition of catecholamine secretion from chromaffin cells evoked by cholinergic agonists or by K+ at a depolarizing concentration (7). Here the effects of chromostatin on catecholamine secretion induced by either 30 μM veratridine, 100 μM histamine, or 10 μM Ca2+ ionophore X537A were compared. Fig. 1 shows that chromostatin produced a dose-dependent inhibition of catecholamine release evoked by carbamoylcholine or by direct depolarization with 10 μM veratridine. Secretion after 10-min incubation with 100 nM chromostatin was inhibited by 80% in cells stimulated with carbamoylcholine and by 50% in directly depolarized cells. In contrast, chromostatin had no effect on secretion induced by histamine or by the calcium ionophore. Chromostatin inhibition on secretion, thus, depends on the secretagogue used to stimulate the cells, indicating that the observed effects may result from modifications occurring at the receptor level or subsequent to that level.

Effect of Chromostatin on the Cytoplasmic Concentration of Cyclic Nucleotides. In response to cholinergic stimulation, cAMP and cGMP levels in cultured chromaffin cells increased, respectively, from 300 ± 10 to 710 ± 20 fmol per 106 cells and from 252 ± 13 to 362 ± 31 fmol per 106 cells. Chromostatin neither modified the cytosolic cAMP and cGMP concentrations in control cells nor affected their increase in stimulated cells.

Effect of Chromostatin on PKC Activity. We have previously shown that catecholamine release evoked by 59 mM K+ was enhanced in cells preincubated for 30 min with 200 nM phorbol 12-myristate 13-acetate (PMA), an activator of PKC in chromaffin cells (19, 20). Chromostatin inhibited secretion in control and in PMA-treated cells with similar potency (data not shown). Moreover, secretion in response to elevated K+ remained inhibited by chromostatin in chromaffin cells preincubated for 48 hr with 1 μM PMA, although such a treatment decreased membrane-bound PKC activity from 900 ± 79 to 62 ± 7 pmol/min per mg of protein. Similarly, staurosporine, a potent PKC inhibitor, did not affect the inhibitory action of chromostatin on catecholamine release evoked by carbamoylcholine (data not shown).

Chromostatin neither modified the PKC distribution in resting cells nor affected its translocation from cytosol to membranes in cells stimulated with 0.5 mM carbamoylcholine (292 ± 35 vs. 474 ± 54 pmol/min per mg of membrane protein in control and carbamoylcholine-treated cells, respectively). Thus, the inhibitory effects of chromostatin are probably not mediated by a pathway involving PKC activation.

Effect of Chromostatin on PPase Activity. Okadaic acid is a potent and highly specific inhibitor of serine/threonine PPases-1 and -2A (21, 22). Fig. 2 shows that secretion induced by carbamoylcholine or 59 mM K+ was not modified in cells pretreated for 20 min with 100 nM okadaic acid, a concentration known to inhibit both PPase-1 and PPase-2A. However, okadaic acid at 100 nM for 20 min or 50 nM for 4 hr had a dramatic effect on chromostatin-induced inhibition because it completely reversed the inhibitory effect of the peptide on catecholamine release evoked by both secretagogues. Only a partial reversion was obtained in cells treated for 24 hr with 10 nM okadaic acid (Fig. 2).

We also measured 45Ca2+ entry into chromaffin cells stimulated with carbamoylcholine or directly depolarized with high K+ in the presence or absence of chromostatin after preincubation with 100 nM okadaic acid. Fig. 3 shows that 100 nM okadaic acid did not modify Ca2+ uptake in chromostatin-un-treated cells but completely reversed the blocking effect of chromostatin on secretagogue-induced Ca2+ entry. These findings strongly suggest that the inhibitory effect of

![Fig. 1](image1.png)

**Fig. 1.** Effect of chromostatin on the secretagogue-induced catecholamine release from cultured chromaffin cells. Chromaffin cells were incubated for 10 min in Locke’s solution containing chromostatin at the indicated concentrations and then stimulated for 10 min with either 10 μM Ca2+ ionophore X537A (a), 100 μM histamine (b), 30 μM veratridine (c), 59 mM K+ (d), or 0.5 mM carbamoylcholine (e). Results are expressed relative to the net [3H]norepinephrine release obtained in the absence of chromostatin (X±S). Data points are mean ± SEM; n = 3. Each point is the mean of three determinations done on four different cell preparations.

![Fig. 2](image2.png)

**Fig. 2.** Effect of okadaic acid on chromostatin-induced inhibition of catecholamine release from cultured chromaffin cells. Chromaffin cells were either preincubated for 24 hr in culture medium (control cells, a) or in culture medium containing 10 nM okadaic acid for 24 hr (c), 50 nM okadaic acid for 4 hr (d), or 100 nM okadaic acid for 20 min (f). Cells were then incubated for another 10 min in Locke’s solution containing chromostatin as indicated. Cells were subsequently stimulated for 10 min with 0.5 mM carbamoylcholine in Locke’s solution. Results are expressed relative to the net [3H]norepinephrine release obtained without chromostatin and okadaic acid (0.5 mM carbamoylcholine, 20.6 ± 0.6%). Each point is the mean ± SEM of three determinations done on two different cell preparations.
Fig. 3. Effect of okadaic acid on chromostatin-induced inhibition of secretagogue-evoked $^{45}$Ca$^{2+}$ uptake. Chromaffin cells were preincubated for 20 min in Locke's solution (control cells) or in Locke's solution containing 100 nM okadaic acid (O.A.) and then incubated for another 10 min with or without 30 nM chromostatin (ChS). Cells were subsequently stimulated for 30 sec with Locke's solution (resting cells) or Locke's solution containing either 0.5 mM carbamoylcholine (CCh 0.5 mM) or 59 mM K$^+$ (K+$^+$ 59 mM) in the presence of $^{45}$Ca$^{2+}$ at 1.5 $\mu$Ci per 200 $\mu$L. Each point is the mean (± SEM) of three determinations done on two different cell preparations.

Chromostatin on Ca$^{2+}$ uptake and catecholamine release may be mediated by a PPase.

Table 1 shows the effects of several CGA-derived peptides on soluble PPase activity. Preincubation of chromaffin cells for 10 min with 100 nM chromostatin increased soluble PPase activity by 2-fold. In contrast, pancreastatin, CAP-14, and rat chromostatin, three CGA-derived peptides that do not inhibit catecholamine release from bovine chromaffin cells (11), had no effect on soluble PPase activity.

Incubation of cells with increased chromostatin concentration produced a dose-dependent increase in the soluble PPase activity, whereas there was no apparent change in membrane-associated PPase activity (Fig. 4). Maximal activation (128%) of soluble enzyme was observed with 10 nM chromostatin, a concentration at which a plateau was reached. It is noteworthy that the concentration curve for PPase activation is very similar to that obtained for catecholamine release (Figs. 1 and 2). No direct effect of chromostatin either on soluble PPase or particulate PPase in chromaffin cells or on PPase-2A purified from bovine heart could be observed.

Characterization of Chromostatin-Sensitive PPase. Okadaic acid is used as a tool to discriminate type-1 and -2 PPases (23). Fig. 5 shows the influence of enzyme concentration on the inhibition by okadaic acid of the PPase activity from the

![Graph](https://example.com/graph.png)

**FIG. 4.** Effect of chromostatin concentration on cytosolic and particulate chromaffin-cell PPase activity with or without okadaic acid. Cells were preincubated for 10 min in Locke's solution with bovine chromostatin as indicated. Cells were then lysed, and extracts were prepared as described. The supernatant (●, ○) diluted 25-fold and particulate extracts (●, ○) diluted 20-fold were assayed for PPase activity by using phosphohistone as a substrate without (●, ○) or with (●, ○) 100 nM okadaic acid. Each point is the mean (± SEM) of three determinations done on two different cell preparations.

**FIG. 5.** Influence of enzyme dilution on inhibition by okadaic acid of chromaffin-cell cytosolic PPase activity. Cells were incubated for 10 min in Locke's solution, lysed, and centrifuged; the supernatant was assayed at different dilutions for PPase activity by using phosphohistone as substrate. Undiluted (○) cytosol supernatant contained 1 mg of protein per ml [measured with the Bradford assay (24)]; dilution factors were 3-fold (●), 10-fold (○), 20-fold (●), 50-fold (○), and 100-fold (■). IC$_{50}$ values were found to depend on dilution—i.e., enzyme concentration, a characteristic of PPase-2A.

Table 1. Effect of chromostatin and okadaic acid on total soluble PPase activity from chromaffin cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>PPase activity, pmol/min per 10$^6$ cells</th>
<th>Relative, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cells</td>
<td>3.54 ± 0.04</td>
<td>100</td>
</tr>
<tr>
<td>Bovine chromostatin</td>
<td>7.63 ± 0.05</td>
<td>215</td>
</tr>
<tr>
<td>Rat chromostatin</td>
<td>3.51 ± 0.02</td>
<td>99</td>
</tr>
<tr>
<td>Pancreastatin</td>
<td>3.56 ± 0.01</td>
<td>101</td>
</tr>
<tr>
<td>CAP-14</td>
<td>3.50 ± 0.02</td>
<td>99</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>0.16 ± 0.01</td>
<td>4.5</td>
</tr>
<tr>
<td>Bovine chromostatin + okadaic acid</td>
<td>0.17 ± 0.01</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Chromaffin cells were preincubated for 20 min in Locke's solution with or without 1 $\mu$M okadaic acid and then incubated for 10 min with 100 nM bovine or rat chromostatin, pancreastatin, or CAP-14. Cells were then lysed and centrifuged; the supernatant was assayed for PPase activity against phosphohistones. Experiments were done on three different cell preparations; triplicate determinations were done on the same cell preparation. Data are given as means (± SEM) of all determinations.
soluble fraction of chromaffin cells: the IC50 for okadaic acid inhibition increased with increased enzyme concentrations, a phenomenon characterizing PPase-2A (23). The observed IC50 (as low as 0.5 nM) is consistent with the PPase being of type 2A, as opposed to type 1, for which much higher okadaic acid concentration is required (23). Moreover, >85% of the soluble PPase activity was abolished by 0.1 μM okadaic acid, suggesting that type 2A is largely predominant in chromaffin-cell cytosol. Treatment of cells with 100 nM chromostatin did not modify the IC50 for okadaic acid inhibition (Fig. 6).

DISCUSSION

The function of CGA, the major soluble component of secretory granules in both adrenal medullary chromaffin cells and in many other endocrine-cell types (1) appears to be that of a prohormone precursor of several biologically active peptides (2–11). Our previous work has shown that CGA-derived peptides exert a negative-feedback control on chromaffin-cell secretory activity (6), and the active peptide has been purified and partially sequenced (7). Chromostatin, a corresponding synthetic peptide composed of 20 amino acids produces a dose-dependent inhibition of the catecholamine secretion evoked by cholinergic agonists (7, 11). Using radiolabeled chromostatin, we have demonstrated the existence of specific receptors for chromostatin on chromaffin cells grown in primary culture (11). The purpose of the present study was to investigate the intracellular pathways coupled to activation of the chromostatin receptor.

Pertussis toxin, which specifically blocks certain G proteins and potentiates the secretory response in chromaffin cells (13), did not modify chromostatin inhibition (data not shown), excluding a coupling with pertussis toxin-sensitive G proteins. In addition, we did not find any modification of cyclic nucleotide levels or PKC activity alteration in cells treated with chromostatin, ruling out involvement of protein kinase A, protein kinase G, and PKC.

In contrast, we have found that the stimulation of the chromostatin receptor selectively activates a cytosolic PPase activity in chromaffin cells. The inhibitory effects of chromostatin on secretagogue-induced Ca2+ uptake and catecholamine secretion in cultured chromaffin cells are completely reversed by okadaic acid, a potent and specific inhibitor of several serine/threonine PPases, demonstrating further that the effects of chromostatin may be mediated by activation of PPases. No direct effect of chromostatin on cytosolic, particulate, or purified PPases was seen, suggesting that an indirect mechanism is responsible for the activation. However a direct mechanism of activation requiring the integrity of the membrane architecture cannot be ruled out.

The PPase type involved in this phenomenon was next characterized. Serine/threonine PPases have been divided into type 1 and type 2, depending on their ability to dephosphorylate preferentially the β or α subunit of phosphorylase kinase (25). Recently, a type 3 PPase has been described, but it can be ruled out because this type, although sensitive to okadaic acid, is associated with the membrane fraction (26); we found no PPase stimulation in the particulate fraction after treatment of chromaffin cells with chromostatin. Type 2 PPases can be subdivided into three distinct enzymes, PPase-2A, PPase-2B, and PPase-2C, the latter two showing an absolute requirement for Ca2+ and Mg2+ respectively, and being insensitive to nanomolar concentrations of okadaic acid. As PPase activation with chromostatin occurred in the absence of any added metal ion (Fig. 5) and was inhibited by okadaic acid, type 2B and 2C PPases can be excluded. Type 2A PPase, rather than type 1 PPase, appears to be the PPase involved in the chromostatin effect because (i) okadaic acid-induced inhibition depends on enzyme concentration and (ii) the IC50 value in both control and chromostatin-stimulated cells is in the nanomolar range. Type 2A, as shown from the okadaic acid-inhibition curve, is largely predominant in the cytosolic fraction of chromaffin cells, in agreement with a previous study (27).

What is the target of this PPase type 2A, which once activated by chromostatin inhibits catecholamine secretion from chromaffin cells? From the lack of chromostatin effect on ionophore- and on histamine-induced secretion, the chromostatin-activated PPase is likely to act at a step before exocytosis. The observation that secretion evoked by depolarizing concentrations of K+ is only partially inhibited by chromostatin, whereas nicotine-induced secretion is totally blocked suggests that dephosphorylation probably occurs on multiple sites.

Activity of many receptors has been shown to depend on their phosphorylation state (28–30). Although the chromaffin-cell acetylcholine receptor has not been studied so far, it is probable that its activity is under the control of a phosphorylation/dephosphorylation cycle. Therefore PPase type 2A may dephosphorylate a key component regulating receptor activity. It is noteworthy that chromostatin has no effect on histamine-evoked secretion, an indication that the histamine receptor is probably not a target of the PPase.

Because chromostatin partially blocks high K+-evoked secretion and reduces Ca2+ entry in stimulated cells as shown here and inhibits L-type calcium currents (11), PPase activation may also affect voltage-gated calcium channels of the L-type. These L-type calcium channels are recruited on stimulation of cells with nicotine but not with histamine (31). Several studies have demonstrated that the activity of voltage-sensitive calcium currents can be modulated by cyclic nucleotide-dependent pathways and/or PKC-dependent phosphorylation (32–36). Armstrong and Eckert (34) have demonstrated that the dihydropyridine-sensitive L-type calcium channels in GH3 cells must be phosphorylated to open when the membrane is depolarized. In the bovine chromaffin cell, phosphorylation by protein kinase A seems to lead to activation of the L-type calcium channel (37). Moreover, dephosphorylation inactivates the calcium channels by leav-
ing them in a state in which they do not open in response to membrane depolarization (34, 38). The role of PPase-2A in modulating ionic channel-gating kinetics has been recently described in rat brain (39) and cardiac muscle (40). From these and other studies on channels in living tissue and on channels reconstituted in artificial bilayers (41-43), it has been proposed that calcium-channel phosphorylation/ dephosphorylation cycle operates continuously and that channel gating and Ca\(^{2+}\) influx can be altered by modulating either the kinase or the phosphatase portion of the cycle. Here such a negative regulation of the L-type calcium channel through dephosphorylation by PPase type 2A might operate in response to stimulation of the receptor to chromostatin.

In conclusion, chromostatin inhibits catecholamine secretion from chromaffin cells by activating a soluble PPase type 2A. The targets of this enzyme are not yet characterized but are likely to be multiple, including plasma-membrane receptors and/or L-type calcium channels.

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