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Influence of salmeterol and benzalkonium chloride on G-protein-mediated exocytotic responses of rat peritoneal mast cells

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Abstract

The long-acting β_2-adrenoceptor agonist salmeterol and the invert soap benzalkonium chloride share physicochemically important structures, namely a polar head group and a long aliphatic chain. Low concentrations of benzalkonium chloride have been shown to inhibit exocytotic responses in rat peritoneal mast cells by selectively interacting with heterotrimeric G-proteins of the G_i-type. The present study investigates whether salmeterol inhibits, independently of β-adrenoceptors, exocytotic responses of rat peritoneal mast cells induced by the direct agonists at G-proteins mastoparan or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS). Exocytosis was studied by secretion assays ([^{3}H]5-hydroxytryptamine ([^{3}H]5-HT)-release) using intact, streptolysin O-permeabilised or metabolically inhibited (antimycin, deoxyglucose) rat peritoneal mast cells. Both amphiphilics, salmeterol, and benzalkonium chloride, dose-dependently exerted biphasic effects on mastoparan-induced [^{3}H]5-HT release in intact mast cells. In contrast to benzalkonium chloride, the dose–response curves for secretostatic and celltoxic effects of salmeterol markedly overlapped. Similar to benzalkonium chloride, salmeterol in non-cytotoxic concentrations (1–25 μg/ml) dose-dependently inhibited exocytosis induced by mastoparan (intact cells) or GTPγS (permeabilised cells). These findings indicate a direct, adrenoceptor-independent affection of G proteins by salmeterol in mast cells.

Keywords: Salmeterol; Benzalkonium chloride; Mast cell; G-protein; β_2-Adrenoceptor; Asthma

1. Introduction

Inhalative β_2-adrenoceptor agonists are first-line drugs for the treatment of bronchoconstriction and asthma (Lipworth, 1999). Among the different β_2-adrenoceptor agonists introduced into clinical praxis, salmeterol has reached a prominent position because of its advantageous longer duration of action. Additionally, in vitro investigations suggested that salmeterol may exert independently of β_2-adrenoceptors some beneficial anti-inflammatory effects (Brogden and Faulds, 1991). Structurally, salmeterol is an amphiphillic compound consisting of a polar β_2-adrenoceptor agonistic saligenin moiety and a long aliphatic tail structure. Ample evidence indicates that the long duration of action of salmeterol is due to the insertion of the aliphatic tail structure into biological membranes, where it probably binds to a certain binding site, termed ''exosite'', which is located beneath or at the β_2-adrenoceptor protein. As a consequence of this process, the interaction of the β_2-adrenoceptor agonistic saligenin moiety with its site of action is prolonged (Green et al., 1996; Coleman et al., 1996). The amphiphilic invert soap benzalkonium chloride comprises a quaternary polar ammonium group and a lipophilic tail structure of comparable length to the aliphatic moiety of salmeterol (see Fig. 1). Low concentrations of benzalkonium chloride have been shown to selectively inhibit heterotrimeric G-proteins of the G_i-type (Read et al., 1982; Higashijima et al., 1990). An effect which has been exceptionally well characterized in rat peritoneal mast cells. In other cell systems, interactions between benzalkonium chloride and other heterotrimeric G-proteins have been described (Patarca and Fletcher, 1995). The structural analogy between both amphiphilics raised the question, whether salmeterol interacts β_2-adrenoceptor-in-

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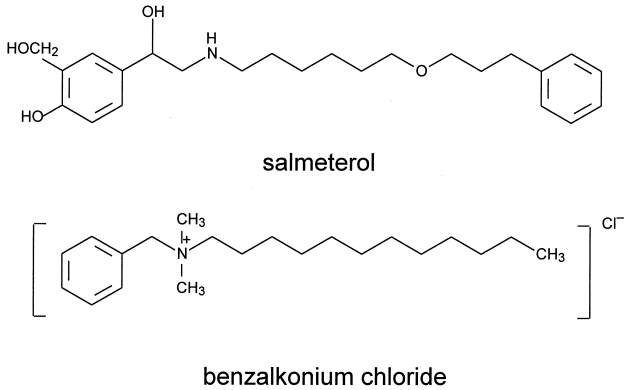

Fig. 1. Chemical structures of salmeterol and benzalkonium chloride[12].

dependently with heterotrimeric G-proteins. Therefore, the influence of salmeterol and benzalkonium chloride on exocytotic serotonin release in rat peritoneal mast cells, stimulated by the direct G-protein activators mastoparan or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) was compared. Mastoparan has previously been shown to stimulate, receptor-independently, a pertussis toxin-sensitive, phospholipase C-activating G-protein of the G_i-type in rat peritoneal mast cells, mediating its exocytotic effect (Mousli et al., 1989; Higashijima et al., 1990; Fischer et al., 1993). In permeabilized rat mast cells, the slow-hydrolyzable GTP-analogue GTPγS has also been frequently used to stimulate exocytosis by direct activation of small and heterotrimeric G-proteins (Howell and Gomberts, 1987; Aridor et al., 1993; Price et al., 1995; Chahdi et al., 1998).

Despite the fact that β_2- and β_1-adrenoceptors have been identified in rat peritoneal mast cells (Marquardt and Wasserman, 1982; Masini et al., 1982), the present study revealed no influence of isoprenaline or propranolol on mastoparan-induced [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT)-release, even when used in high concentrations. It has previously been shown that permeabilized rat peritoneal mast cells rapidly loose their ability to respond to external agonist, probably due to washout of cytosolic constituents (Howell and Gomberts, 1987; Pinxteren et al., 1998). Indeed, under our experimental conditions, 1.5 min after addition of 1.0 U/ml streptolysin O, mastoparan failed to stimulate [^{3}H]5-HT release. Therefore, both models of agonist-induced exocytosis used in the present study can be considered as functionally β_2-adrenoceptor-independent and are suitable to study the β_2-adrenoceptor-independent actions of salmeterol. Additionally, the thresholds for the toxic effects of salmeterol and benzalkonium chloride were monitored by pretreatment of rat peritoneal mast cells with metabolic inhibitors (antimycin, deoxyglucose) in a glucose-free medium, a measure that has been shown to completely prevent the exocytotic but not the membranolytic release of mast cell mediators (Mohr and Fewtrell, 1990).

2. Materials and methods

2.1. Solutions and reagents

Benzalkonium chloride ($C_{12}H_{25}N(CH_3)_2$–C_7H_7Cl and $C_{14}H_{25}N(CH_3)_2$–C_7H_7Cl) and bovine serum albumin were obtained from Sigma (Deisenhofen, Germany). The following substances were purchased from different manufacturers, as indicated: Salmeterol (Glaxo Wellcome, UK or Sigma), mastoparan H-Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2 (Bachem, Germany), GTPγS (Boehringer Mannhein, Germany), streptolysin O (RBI, Germany), and [^{3}H]5-HT (NEN, Germany).

2.2. [^{3}H]5-HT release experiments in intact cells

Rats (200–400 g) were anaesthetised and decapitated. Mast cells were obtained by peritoneal lavage with a modified Krebs–Ringer–Hepes (KRH) buffer (NaCl 137.0 mM, KCl 2.7 mM, $CaCl_2$ 0.3 mM, $MgCl_2$ 1.0 mM, NaH_2PO_4 0.4 mM, HEPES 10.0 mM, Glucose 5.6 mM, 0.2% bovine serum albumin; pH 7.3) and purified by subsequent centrifugation through a 0.2/40% (wt/vol) bovine serum albumin-gradient (100 g, 5 min, 4°C), as previously described (Mousli et al., 1989; Seebeck et al., 1998). This method yields mast cells at an average purity above 90%, as evidenced by alcian blue staining (data not shown). Subsequently, cells were resuspended in KRH-buffer and incubated for 2 h in the presence of 1 μCi/ml [^{3}H]5-HT in a shaking water bath at 37°C. After further wash, cells were resuspended in KRH or a potassium-rich permeabilization buffer (see below) and stimulated as indicated. Secretion was assessed as the relative amount of intracellular radioactive tracer released into the extracellular medium. Experiments with a spontaneous [^{3}H]5-HT release above 5% were excluded. All concentrations were determined in duplicate.

2.3. [^{3}H]5-HT release experiments in streptolysin O-permeabilized cells

Rat peritoneal mast cells were permeabilized by means of the pore forming toxin streptolysin O according to the instructions given by Howell and Gomberts (1987), with slight modifications. Briefly, purified mast cells, prepared as described above, were dissolved in potassium-rich, albumin-free permeabilization-buffer (NaCl 30.0 mM, KCl 110 mM, NaH_2PO_4 0.4 mM, Hepes 15 mM). Consecutively, 150 μl aliquots of this cell suspension were added into test vials pre-filled with streptolysin O (1.0 U/ml) and a Ca^{2+}/EGTA-buffer (EGTA 2.5 mM). The EGTA-buffer was added in order to adjust micromolar concentrations of free Ca^{2+} (for preparation, see Neher, 1988). Consecutively, the permeabilization process was started by

transferring the cells rapidly into a shaking water bath (37°C); test compounds (GTPγS, salmeterol, etc.) were added at different time points as indicated, to a final incubation volume of 300 μl. The incubations were terminated after 8 min by transferring the cells on ice. Under standard conditions, no metabolic inhibitors were used. Because benzalkonium was used as a mixture of benzalkonium$_{12}$ and benzalkonium$_{14}$ (both derivatives have been shown to possess G_i-inhibitory activity; Fischer et al., 1993), concentrations were given as wt/vol. For comparability reasons, salmeterol concentrations were similarly expressed.

2.4. Statistical analysis, curve fittings

Results are given as means ± S.E.M. The statistical significance of differences was analysed by the two-sided Wilcoxon test for matched pairs and P values of less than 0.05 were considered to be significant. Agonist and antagonist concentration–response curves were fitted to a four parameter logistic equation through computer-assisted curve fitting (Prism 2, GraphPad software, San Diego, USA). The equation fitted was

$$\text{Response} = E_{\min} + (E_{\max} - E_{\min})/(1 + 10^{(\log EC_{50} - X)n_H}),$$

where $E_{\min}$ is the basal response, $E_{\max}$ is the maximal stimulation, X is the agonist concentration, and n_H is the Hill coefficient.

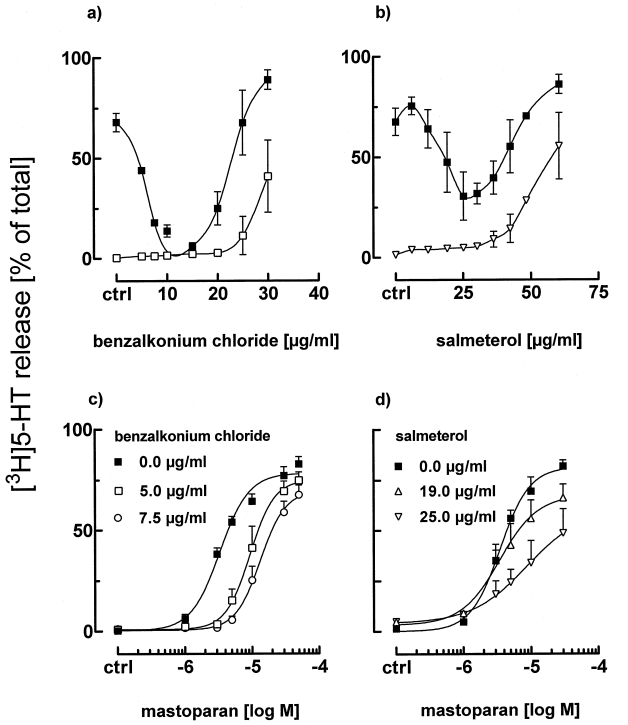

Fig. 2. Dose-dependent effect of benzalkonium chloride (a; $n = 3$) or salmeterol (b; $n = 5$) on [^{3}H]5-HT-release from purified rat peritoneal mast cells, stimulated for 10 min with (closed symbols) or without (open symbols) 10 μM mastoparan. (c and d) Dose-dependent effect of mastoparan on [^{3}H]5-HT release in the absence or presence of different concentrations of benzalkonium chloride (c; $n = 5$) or salmeterol (d; $n = 6$) in purified rat peritoneal mast cells. Results are expressed as means ± S.E.M.

3. Results

3.1. Influence of salmeterol and benzalkonium chloride on mastoparan-induced secretory responses in intact rat peritoneal mast cells

Preincubation (3.0 min) of non-permeabilized mast cells with low concentrations of salmeterol (< 30.0 μg/ml) or benzalkonium chloride (< 20.0 μg/ml) dose-dependently reduced the secretory effect of 10.0 μM mastoparan (incubation time: 10.0 min); at higher concentrations both compounds stimulated the release of [^{3}H]5-HT (Fig. 2a,b). The exocytotic mastoparan-response was completely inhibited at benzalkonium chloride-concentration below those eliciting a [^{3}H]5-HT release (IC_{50}: 6.0 μg/ml, IC_{100}: 15.0 μg/ml; $n = 3$). With salmeterol, however, the highest non-stimulatory concentration (25 μg/ml) reduced the mastoparan-effect by only 59% (secretion [% of total]: mastoparan, 67.7 ± 3.0; mastoparan + 25 μg/ml salmeterol, 30.6 ± 5.4; $n = 5$; Fig. 2b).

Additionally, the influence of salmeterol and benzalkonium chloride on secretory responses induced by different concentrations of mastoparan were assessed. Three mastoparan dose–response curves (1–30 μM) in the presence of different concentrations of salmeterol (0.0, 19.0, 25.0 μg/ml) or benzalkonium chloride (0.0, 5.0, 7.5 μg/ml) were generated. The mastoparan log dose–response curves could be described by a logistic function (EC_{50}, 3 μM; Hill coefficient (n_H) = 2.0; $n = 5$; Fig. 2c). Preincubation with benzalkonium chloride (0.0, 5.0 and 7.5 μg/ml) induced a nearly parallel rightward shift of the mastoparan dose–response curves (EC_{50}, 3 μM; $n_H = 2.0$; EC_{50}, 9 μM; $n_H = 2.4$; EC_{50}, 13 μM; $n_H = 2.3$; $n = 7$; Fig. 2c). In contrast, preincubation with 19.0 or 25.0 μg/ml salmeterol markedly changed the slope of the mastoparan dose–response curves (n_H, 1.5 and 1.0 rs., $n = 6$, Fig. 2d).

Within the same series of experiments as described in Fig. 1a, isoprenaline (10^{-4} M) neither stimulated nor did the adrenoceptor agonist inhibited the mastoparan-response ($n = 5$; data not shown). The presence of propranolol (10^{-6}–10^{-4} M) did not change the inhibitory effect of 25 μg/ml μM salmeterol. At concentrations above 10^{-4} M, propranolol itself promoted the release [^{3}H]5-HT in rat peritoneal mast cells ($n = 5$, data not shown).

3.2. Influence of salmeterol and benzalkonium chloride on GTPγS-induced secretory responses in streptolysin O-permeabilized rat peritoneal mast cells

In a typical permeabilization experiment, different compounds were applied after the start of the action of streptolysin O (time point: 0 min) in the following order: 2 min salmeterol or benzalkonium chloride, 4 min ATP (1 mM), 5 min GTPγS. In streptolysin O-permeabilized mast cells, benzalkonium chloride inhibited the 10 μM GTPγS-induced secretory response significantly, with high potency, in a dose-dependent manner (IC_{100}, 2.0 μg/ml; $P < 0.001$; $n = 11$; Fig. 3a). At concentrations above 5 μg/ml, benzalkonium chloride promoted the release of [^{3}H]5-HT (data not shown). Under similar experimental conditions also salmeterol (1–60 μg/ml) dose-dependently reduced the exocytotic response induced by 10 μM GTPγS in streptolysin O-permeabilised rat peritoneal mast cells (IC_{50}, 30 μg/ml; $n = 4$; Fig. 3b).

3.3. Influence of metabolic inhibition on [^{3}H]5-HT-release induced by salmeterol, benzalkonium chloride, and mastoparan

In intact rat peritoneal mast cells, the stimulatory effects of benzalkonium chloride and salmeterol could not be inhibited by preincubating the cells for 30 min, at 37°C in a glucose-free medium, in the presence of the metabolic inhibitors 2-deoxy-D-glucose (5.6 mM) and antimycin A (0.1 μM). In these experiments, salmeterol at concentrations > 30 μg/ml and benzalkonium chloride (≧ 10 μg/ml) dose-dependently stimulated the release of [^{3}H]5-HT in rat peritoneal mast cells pretreated with metabolic inhibitors, while corresponding mastoparan-controls were markedly reduced by metabolic inhibition ([% of total]: 59.5 ± 0.7 vs. 6.7 ± 0.7; $n = 2$).

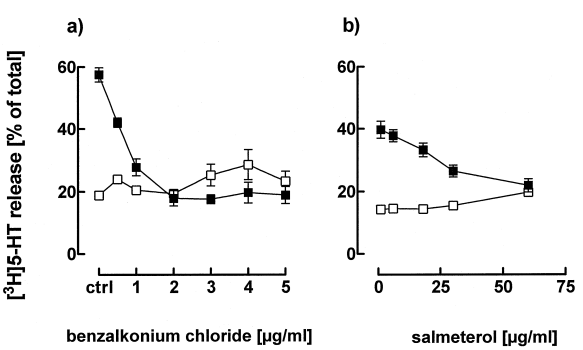

Fig. 3. Dose-dependent effect (preincubation time: 3 min) of benzalkonium chloride (a; $n = 11$) or salmeterol (b; $n = 4$) on [^{3}H]5-HT release from streptolysin O-permeabilized rat peritoneal mast cells, stimulated with (closed symbols) or without (open symbols) 10 μM GTPγS. Results are expressed as means ± S.E.M.

4. Discussion

The long-acting β_2-adrenoceptor agonist salmeterol and the disinfectant benzalkonium chloride show remarkable structural similarities. Both compounds are amphiphilic molecules with a polar head group and long aliphatic tail structure of comparable length. The aliphatic tail structure of both compounds is assumed to insert into biological membranes. Low, non-cytotoxic, concentrations of benzalkonium chloride have been shown to modulate the function of a variety of heterotrimeric G-proteins, e.g. in rat peritoneal mast cells, low concentrations of benzalkonium chloride competitively inhibit exocytosis induced by different direct activators (e.g. compound 48/80, mastoparan) of heterotrimeric G-proteins of the G_i-type (Read and Kiefer, 1979; Read et al., 1982; Higashijima et al., 1990; Fischer et al., 1993; Chahdi et al., 1998; Freissmuth et al., 1999). Therefore, the aim of the present study was to assess, if salmeterol shares the property to inhibit G-proteins via receptor-independent, direct mechanisms. To this, the influence of salmeterol and benzalkonium chloride on mast cell exocytotic responses induced by mastoparan and the slowly hydrolyzable GTP-analogue GTPγS were compared. Additionally, the cytotoxic properties of both compounds were assessed by metabolic inhibiton of rat peritoneal mast cells, a measure which has been previously shown to prevent the exocytotic but not the toxic release of mast cell granule contents (Mohr and Fewtrell, 1990).

4.1. Effect of salmeterol and benzalkonium chloride on receptor-independent G-protein-mediated exocytosis in rat peritoneal mast cells

In accordance with previous studies, benzalkonium chloride inhibited the mastoparan- and GTPγS-induced exocytotic response in a dose-dependent manner. Preincubation of rat peritoneal mast cells with 5.0 and 7.5 μg/ml benzalkonium chloride led to a parallel rightward-shift of the mastoparan dose–response curve and to an insurmountable decrease of the mastoparan maximal effect (Fig. 2c). On the basis of this functional data, benzalkonium chloride appears as a non-competitive inhibitor of G_i-proteins. In isolated membrane preparations, Higashijima et al. (1990) have shown that benzalkonium chloride and mastoparan behave as competitive antagonists at the G-protein-level. These contradictory results might be explained by the localization of the target G-proteins ''behind'' a plasma membrane, preventing the development of equilibrium conditions. Therefore, our results from intact cells, by definition, are not suitable to judge the nature of the observed antagonistic effects of salmeterol and benzalkonium chloride (Kenakin, 1997). Due to the time-dependent dynamic actions of streptolysin O, the same restrictions hold true for our findings from permeabilised cells. Like benzalkonium chloride, also salmeterol in a

concentration range between 5 and 25 μg/ml reduced the exocytotic mastoparan-response in an insurmountable manner, whereas higher concentrations of the beta-agonist stimulated the release of [^{3}H]5-HT from intact cells via metabolism-independent, nonexocytotic (cytotoxic) mechanisms. Divergent from benzalkonium chloride, the dose–response curves for the secretostatic, and the cytotoxic effect of salmeterol markedly overlapped. The GTPγS-induced exocytotic response in streptolysin O-permeabilised cells was potently inhibited by very low concentrations (1 μg/ml) of benzalkonium chloride and also, but less potently, by salmeterol (Fig. 3a,b). These findings indicate that salmeterol and benzalkoium chloride are able to inhibit exocytotic responses induced by direct activators of G-proteins in rat peritoneal mast cells. Accordingly, Lau et al. (1994) reported that salmeterol in concentrations above 10^{-5} M (≈ 4 μg/ml), but no other β_2-adrenoceptor agonist, inhibited histamine release from rat peritoneal mast cells challenged by cross-bridging of high-affinity immunglobulin E-receptors. It is important to note, that despite the fact that β_1- and β_2-adrenoceptors have been identified functionally and in binding studies in rat peritoneal mast cells, in the present study, the mastoparan-effect was neither influenced by preincuabtion with a β-adrenoceptor agonists (isoprenaline) nor by the antagonist propranolol. Therefore, it can be concluded that the investigated acute biological responses are not under the control of β-adrenoceptors.

4.2. Cytotoxic effects of salmeterol and benzalkonium chloride

In higher concentrations, benzalkonium chloride and salmeterol stimulated the release of [^{3}H]5-HT in intact cells as well as in streptolysin O-permeabilized cells. In order to verify that these stimulatory effects are of cytotoxic nature, rat peritoneal mast cells were incubated in glucose-free medium in the presence of two metabolic inhibitors (antimycin A, deoxyglucose), a measure which has been previously shown to prevent exocytotic responses of these cells (Mohr and Fewtrell, 1990). As expected, the salmeterol- and benzalkonium chloride-induced release of [^{3}H]5-HT was not reduced by metabolic inhibition of rat peritoneal mast cells, while mastoparan-controls were clearly inhibited (see Section 3.3). It can be concluded that the release of [^{3}H]5-HT from rat peritoneal mast cells stimulated by salmeterol and benzalkonium chloride is due to a lytic and not to an exocytotic process. With regard to benzalkonium chloride, similar conclusions have been drawn in previous studies (Read and Kiefer, 1979).

4.3. Adrenoceptor-independent effects of salmeterol in other studies

Salmeterol in μM-concentrations has been shown to induce a number of potentially beneficial (e.g. anti-inflammatory) as well as detrimental (proinflammatory, proarrhythmic) β_2-adrenoceptor-independent effects in vitro (Baker et al., 1994; Prevost et al., 1997; Chong et al., 1998; Ezeamuzie and Al-Hage, 1998). The extent to which these effects contribute to the therapeutic actions of salmeterol is unclear. It has to be reminded that the β_2-adrenoceptor-mediated effects of salmeterol occur in the pico to nanomolar range (Butchers et al., 1991; Green et al., 1996; Chong et al., 1998). The typical concentration of salmeterol-xinafoat in commercial aerosol preparations is 2.4×10^{-4} M, therefore only in the case of a defective distribution or clearance of the drug, β_2-adrenoceptor-independent actions of salmeterol may become clinically relevant.

In conclusion, salmeterol and benzalkonium chloride dose-dependently exerted biphasic effects on mastoparan-induced [^{3}H]5-HT release in intact rat peritoneal mast cells. In contrast to benzalkonium chloride, the secretostatic and the cytotoxic dose–response curves of salmeterol markedly overlapped. Both amphiphilic compounds in non-cytotoxic concentrations reduced the exocytotic response stimulated by the direct activators of G-proteins, mastoparan (intact cells), and GTPγS (permeabilised cells). These findings indicate, that salmeterol in subcytotoxic concentrations can attenuate G protein-function via adrenoceptor-independent mechanisms in rat mast cells.

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