Bay K 8644 enhances the outflow of [3H]-noradrenaline and [3H]-DOPEG from isolated rat atria

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Summary. The effect of Bay K 8644 (a dihydropyridine Ca^{2+}-channel activator), was examined on spontaneous and stimulus-evoked release of tritium from isolated rat atria prelabelled with [3H]-noradrenaline. Bay K 8644 (3 μmol/l) significantly increased atrial rate from 206 ± 7 to 259 ± 9 beats·min^{-1} ($P < 0.05$) and also tritium outflow (expressed as fractional rate of loss in $min^{-1} \times 10^3$) from 6.49 ± 0.35 to 8.61 ± 0.74 ($P < 0.05$). Neither the maximal rate nor the overflow of tritium induced by stimulation of sympathetic nerve terminals was changed by the compound. The increase in basal tritium outflow produced by Bay K 8644 was calcium-dependent. However, it could not be antagonized by nitrendipine. The overflow of tritium induced by Bay K 8644 consisted mainly of 3,4-dihydroxyphenylglycol ([3H]-DOPEG), indicating that the compound produces a leakage from the storage vesicles of sympathetic nerve terminals of the isolated rat atria.

Key words: Bay K 8644 — Rat atria — Tritium efflux — Adrenergic nerve endings — [3H]-noradrenaline

Introduction

The release of neurotransmitters depends on the entry of Ca^{2+} ions from the extracellular fluid (Kirperkar and Misu 1964; Katz and Miledi 1968) through voltage-operated channels (Katz and Miledi 1968; Nachshen and Blaustein 1980). Three subtypes (L, T and N) of Ca^{2+} channels have been described by Nowycky et al. (1985) in neuronal cells. These subtypes of voltage-dependent Ca^{2+} channels are distinguished by several properties, including their different sensitivities to 1,4-dihydropyri-

dines. The L-type Ca^{2+} channel, but neither the T-nor the N-type, is sensitive to the dihydropyridine Ca^{2+}-channel blockers and activators. In a large variety of neuronal preparations the evoked release of neurotransmitters has generally proved to be insensitive to dihydropyridines despite the presence of high-affinity dihydropyridine-binding sites (Nachshen and Blaustein 1979; Daniell et al. 1983; Miller and Freedman 1984). This might suggest that transmitter release may be controlled by dihydropyridine-resistant Ca^{2+} channels.

Slight modifications in the structure of dihydropyridine antagonists yield compounds like Bay K 8644 that enhance rather than inhibit Ca^{2+} influx in smooth and cardiac muscles (Schramm et al. 1983; Wahler and Speralakis 1984; Thomas et al. 1985). It has been reported that in chick dorsal root ganglion cells Bay K 8644 increased the frequency and the duration of Ca^{2+}-channel openings (Nowycky et al. 1985) and augmented KCl-stimulated release of substance P (Perney et al. 1986; Holz et al. 1988) as well as potentiated the potassium-induced release of [3H]-noradrenaline from cultured PC 12 cells (Shalaby et al. 1984), adrenal medullary cells (García et al. 1984) and brain slices (Middlemiss 1985). We have reported (Camilión de Hurtado et al. 1989) that the positive chronotropic effect of Bay K 8644 in isolated rat atria was partially inhibited by the beta-adrenoceptor antagonist propranolol or by pretreatment with reserpine. These data strongly favor the idea that the chronotropic effect of Bay K 8644 depends at least in part on the release of noradrenaline onto the pacemaker. With the aim of exploring in further detail the action of Bay K 8644 we studied the effect of this compound on the rate of contraction and the release of tritium from isolated rat atria preloaded with [3H]-noradrenaline.

Material and methods

Female Wistar rats of 250—300 g of body weight were used. The animals were anaesthetized with pentobarbitone (80 mg/kg i.p.) and the atrial pair with the cardioaccelerans nerves were dissected free of connective tissue. The preparations were mounted in a 5-ml organ bath hav-

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ing two stainless steel ring electrodes through which the cardioaccelerans nerves were passed. One end of the atrial pair was fixed to a holder and the other was connected to a Statham force transducer. Isometric atrial contractions were recorded by a Hewlett Packard 7712 Recorder to determine atrial rate. The following incubation medium was used (in mmol/l): NaCl 128.3; KCl 4.5; $CaCl_2$ 1.35; $NaHCO_3$ 20.2; NaH_2PO_4 0.4; $MgCl_2$ 1.05; EDTA 0.04; ascorbic acid 0.11; glucose 11.1. Atropine sulphate (final concentration 3 μmol/l) was added to the solution to avoid any influence through activation of muscarinic receptors. The solution was equilibrated with a gas mixture of 5% CO_2 in 95% O_2 and the experiments were carried out at 37 °C. The set up allowed the consecutive measurement of the spontaneous atrial rate and the chronotropic response to electrically induced depolarizations of the cardioaccelerans nerves, as well as the collection of samples of the bathing media.

In 9 of the experiments the cardioaccelerans nerves were stimulated for 20-s periods at different frequencies ranging from 0.2 to 12.8 Hz (supramaximal voltage, 0.5 ms pulse width). The increase in atrial rate induced by nerve stimulation was determined after cessation of the electrical stimulus. The interval between each period of stimulation was 5 min, this time being enough for the atrial rate to return to its basal value. Two consecutive (30 min apart) frequency-response curves were determined in each preparation. In five of these experiments the second frequency-response curve was determined in the presence of 3 μmol/l Bay K 8644 which was added to the bathing solution 20 min before.

In the experiments in which the noradrenaline stores were labelled, the atria were incubated for 30 min with 0.335 μmol/ml of (±)-[7-^{3}H]-noradrenaline (specific activity 15 Ci/nmol; New England Nuclear, Dreieich, FRG). At the end of the incubation with [^{3}H]-noradrenaline several washes with amine-free solution were made according to the following schedule: 5 washes every 2 min, 7 washes every 10 min and 40 washes every 2 min. The efflux of tritium from the noradrenergic nerve terminals of the tissue was determined from the tritium content of the incubation media collected during the last 80 min of washout. While the first 20 min of this observation period (80th–100th min) served to determine the efflux of tritium under control conditions, the last part of this period (100th–160th min) served to determine the effect of drugs on the efflux of tritium. At the end of the experiment the atria were solubilized in 0.5 ml of 1 mol/l hyamine hydroxide at 50 °C overnight. The tritium content of the tissue was determined after neutralization with maleic acid.

Fractional rates of loss (FRL) were calculated as previously described (Camilión et al. 1989); the values expressed by minute were multiplied by 1000 (min^{-1}·10^3). Basal outflow of tritium was expressed as the mean FRL determined during the two collection periods preceding nerve stimulation. The stimulus-induced total overflow of tritium was the sum of the increases in FRL above the spontaneous levels during the period of nerve stimulation and also during the subsequent ones until the ^{3}H outflow returned to basal values. Increases in FRL were determined by subtracting the spontaneous outflow assumed to have occurred during each period from the total amount of tritium collected in each sample.

The cardioaccelerans nerves were electrically stimulated for 80 s after the 91st min (S_1) and after the 121st min (S_2). Each stimulation consisted of 3 consecutive trains of 80 pulses each (4 Hz, 0.5 ms, supramaximal voltage) with a 10-s interval between trains. Bay K 8644 was added to the washing media 20 min before S_2 and continued to be present until the end of the experiment.

In control experiments ($n = 5$) the spontaneous FRL remained virtually constant during the last 80 min of washout. The stimulation-evoked overflow of tritium did likewise not change during this time, since the ratio of the FRL response to S_1 to that of S_2 was 1.01 ± 0.40.

In a group of 4 experiments, Ca^{2+} was omitted from the solution used for washing the atria after labelling the tissues in normal solution.

Samples of some washout media were subjected to column chromatography to determine the contribution of unchanged [^{3}H]-noradrenaline and its tritiated metabolites to total tritium as described by Graefe et al. (1973).

Results are expressed as mean ± SEM. Student's t-test for paired data was used for statistical comparisons. The level of significance was chosen as $P < 0.05$.

The drugs used were (±)-[7-^{3}H]-noradrenaline (New England Nuclear, Dreieich, FRG); Bay K 8644 (Bayer AG, Leverkusen, FRG); nitrendipine (Bayer AG, Leverkusen, FRG); atropine sulphate (E. Merck AG, Darmstadt, FRG); Dowex-50 Wx 4-400 (Sigma Chemical Co., St. Louis, USA); aluminium oxide (E. Merck, Darmstadt, FRG). All other reagents and salts of the bathing solution were of maximal purity grade. Dihydropyridines were diluted with polyethyleneglycol 400 and protected from light exposure.

Results

Figure 1 shows the chronotropic response to increasing frequencies of electrical stimulation of the cardioaccelerans nerves. Under control conditions there was no difference between two consecutive frequency-response curves determined 30 min apart (panel A). Panels B and C show the results obtained when the second frequency-response curve was obtained in the presence of 3 μmol/l Bay K 8644. The compound increased the basal atrial rate by 66 ± 10 beats·min^{-1} ($P < 0.05$), without significantly changing the maximum rate elicited by nerve stimulation (panel B). In consequence the maximum chronotropic response to nerve stimulation in the presence of Bay K 8644 was decreased when it was expressed as the percentage of the maximum response obtained in its absence (panel C).
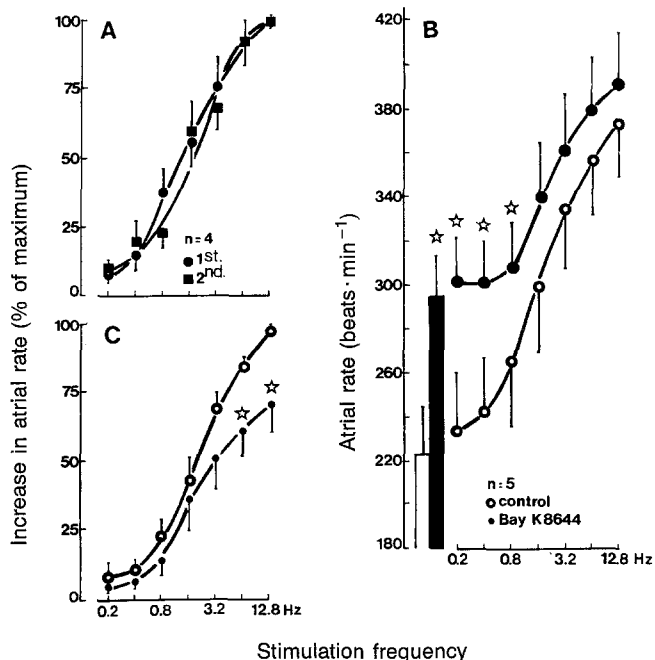

Fig. 1. Chronotropic responses of the atrial pair of the rat to cardioaccelerans nerve stimulation. *Panel A:* Two consecutive frequency-response curves to cardioaccelerans nerve stimulation were determined in control medium 30 min apart. The chronotropic response is expressed as percentage of the maximal increase in rat of the first curve (109 ± 13 beats·min^{-1}, $n = 4$). *Panel B:* The 1st frequency-response curve (*open symbols*) was obtained on atria incubated in control medium. The 2nd curve (*closed symbols*) was obtained after 3 μmol/l Bay K 8644 was added to the medium. *Bars* show values of spontaneous atrial rate in control medium (*open bar*) and in the presence of 3 μmol/l Bay K 8644 (*closed bar*) ($n = 5$). *Panel C:* Same results as in panel B expressed as percentage of the maximal increase in rate obtained in control medium.
* denotes significant differences, $P < 0.05$, paired 't' test

Figure 2 shows the spontaneous and stimulus-induced outflow of tritium from atria previously labelled with [^{3}H]-noradrenaline. Mean values of spontaneous and stimulus-evoked tritium outflow prior to and during the exposure to Bay K 8644 or its solvent are shown in panels A and B, respectively. Exposure to 3 μmol/l Bay K 8644 increased by 33% the basal outflow of tritium. The mean FRL determined during the two collection periods preceding the nerve stimulation were (in min^{-1}·10^3) 6.49±0.35 in the control and 8.61±0.74 during drug exposure ($n = 8$, $P < 0.05$). Exposure to the solvent of the drug did not change basal tritium release. Accordingly, only Bay K 8644 and not the solvent increased the basal atrial rate of contraction (Table 1). By contrast, the stimulus-evoked overflow of tritium was not altered by the compound. The stimulus-induced total tritium overflow was 0.66±0.15% of tissue content under control conditions and 0.58±0.09% in the presence of Bay K 8644 ($n = 9$). Bay K 8644 also failed to change the peak atrial rate attained after nerve stimulation (Table 1). As a consequence, the increase in rate induced by nerve stimulation tended to be lower although the difference was below the level of significance ($P > 0.05$).

The increase in basal outflow of tritium induced by Bay K 8644 was a calcium-dependent process. Figure 2 (panel C) shows that the effect of 3 μmol/l Bay K 8644 was suppressed when calcium was omitted from the medium. However, in a calcium-containing medium nitrendipine failed to antagonize the effect of Bay K 8644. From the 130th min onwards, 5 of the atria belonging to the group of experiments shown in Fig. 2, panel A, were washed with a solution containing 0.1 μmol/l nitrendipine plus 3 μmol/l Bay K 8644. The other 4 atria were washed with a higher nitrendipine concentration (0.3 μmol/l). Neither of these concentrations inhibited the effect of Bay K 8644 on the basal outflow of tritium.

Since the atria washed with Ca^{2+}-free media were quiescent we conducted another set of experiments to explore whether the effect of Bay K 8644 on tritium release was influenced by the rhythmic spontaneous contraction of the myocardium surrounding the adrenergic nerve endings. Thus, tritium outflow was measured in the beating right atrium and in the quiescent left atrium obtained from the same rat. FRL of tritium was determined during the 88th and 89th min after the end of the labelling period and following the incubation with 3 μmol/l Bay K 8644 for 20 min. Basal outflow of tritium (in min^{-1} × 10^3) increased from 7.26±0.77 to 10.67±1.74 in right atrium ($n = 6$, $P < 0.05$) and from 7.20±0.75 to
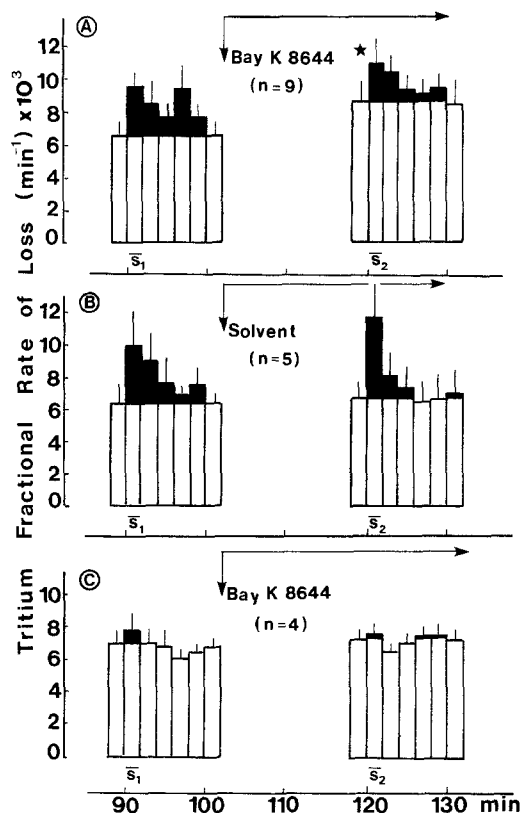

Fig. 2. Spontaneous and stimulus-evoked tritium efflux from the atrial pair labelled with [^{3}H]-noradrenaline. *Ordinate:* Values of FRL are multiplied by 1000. *Abscissa:* time after the end of the labelling period. S_1 and S_2: electrical stimulation of cardioaccelerans nerves each consisting in three consecutive trains of 80 pulses each at supramaximal voltage, 0.5 ms duration, 4 Hz with a 10-s interval between each train. The first open column of each group of columns represents the tritium efflux preceding the period of nerve stimulation. This basal efflux was assumed to occur unaltered during and following the period of nerve stimulation. *Closed areas:* stimulation-evoked release of tritium over baseline spontaneous outflow. The *arrows* indicate the beginning of the exposure of tissues to 3 μmol/l Bay K 8644 (panels A and C) or to the solvent (panel B). In panel C the washout was carried out with Ca^{2+}-free media. * denotes significant differences from pre-drug values, $P < 0.05$, paired 't' test

8.28±0.57 in left atrium ($n = 6$, $P < 0.05$) but there was no significant difference between beating and quiescent atrium.

Table 2 shows the metabolic profile of tritium in the basal outflow and in the Bay K 8644-induced overflow. Under our experimental conditions about 20% of total tritium outflow consisted of unchanged [^{3}H]-noradrenaline. During exposure to Bay K 8644 there was a drastic

Table 1. Rate of beating (in beats·min^{-1}) of isolated rat atria before and during exposure to 3 μmol/l Bay K 8644 or its solvent
Mean values and standard error of atrial rate (beats·min^{-1}). a) Before electrical nerve stimulation; b) peak atrial rate recorded after the period of nerve stimulation (see parameters of stimulation in Fig. 2); c) increases in rate over basal values induced by nerve stimulation. *$P < 0.05$ compared to pre-drug values, paired 't' test

Treatment	Before drug exposure			During drug exposure		
	Basal (a)	After nerve st (b)	Increase in rate (c)	Basal (a)	After nerve st (b)	Increase in rate (c)
Bay K 8644 ($n = 8$)	206± 7	345±10	140±12	259± 9*	352±19	93±18
Solvent ($n = 5$)	210±15	353±27	143±22	201±20	361±17	160±23

Table 2. The effect of Bay K 8644 on [^{3}H]-noradrenaline metabolite pattern

Metabolic distribution of tritium in the bathing media collected during 4 min both, 86 min after the end of the labelling period (control) and following 20 min of exposure to 3 µmol/l Bay K 8644. * Denotes $P<0.05$, paired 't' test between Bay K 8644 values and their corresponding controls

Fractions	Percentage of total radioactivity			
	Control medium ($n = 8$)		Ca^{2+}-free medium ($n = 4$)	
	Control	Bay K 8644	Control	Bay K 8644
Noradrenaline	23.5 ± 6.0	20.4 ± 5.6	12.2 ± 2.6	10.5 ± 1.7
DOPEG	19.7 ± 3.3	38.7 ± 7.4*	9.0 ± 2.2	14.1 ± 2.3
DOMA	11.1 ± 1.8	12.8 ± 2.2	12.2 ± 1.7	12.5 ± 5.4
0-Methylated	52 ± 7	57 ± 9	48 ± 3	41 ± 3
Ratio noradrenaline/DOPEG	1.19 ± 0.21	0.60 ± 0.11*	1.12 ± 0.14	0.77 ± 0.19

increase in the efflux of 3,4-dihydroxyphenyl glicol ([^{3}H]-DOPEG) without changes in the percentage of unchanged [^{3}H]-noradrenaline. Therefore the ratio [^{3}H]-noradrenaline/[^{3}H]-DOPEG decreased. No significant changes in the proportion of 3,4-dihydroxymandelic acid ([^{3}H]-DOMA) and [^{3}H]-0-methylated compounds were detected following the administration of Bay K 8644·Bay K 8644 did not induce changes in the metabolite pattern in the efflux of tritium from atria incubated in a Ca^{2+}-free medium (Table 2).

Discussion

Bay K 8644 induced increases in both the atrial rate and tritium efflux from rat atria preloaded with [^{3}H]-noradrenaline in a Ca^{2+}-dependent manner. These results confirm and extend previous data from our laboratory (Camilión de Hurtado et al. 1989) suggesting the involvement of endogenous catecholamines in the chronotropic effect of Bay K 8644. During the exposure to Bay K 8644 the proportion of [^{3}H]-DOPEG efflux increased while that of [^{3}H]-noradrenaline remained unchanged. In consequence the amount of noradrenaline reaching the receptor sites must have increased since total tritium efflux had increased.

After the loading of sympathetic nerve endings with [^{3}H]-noradrenaline and during the subsequent washout of the tissues with amine-free solution, a spontaneous neuronal efflux of tritium is obtained. Usually, only a small proportion of the radioactivity leaving the tissue is composed of unchanged [^{3}H]-noradrenaline, since [^{3}H]-DOPEG is the main metabolite in the washout media (Adler-Graschinski et al. 1972; Graefe et al. 1973; Steppeler and Starke 1980). Under our experimental conditions about 20% of the total tritium efflux was composed of the unchanged ^{3}H-amine. Although somewhat higher than those values commonly cited in the literature, this value was similar to those reported by Pesce and Adler-Graschinski (1983) under similar experimental conditions.

In the spontaneous neuronal efflux the ratio noradrenaline/DOPEG is usually below unity. Three factors appear to contribute to this: a) extensive intraneuronal deamination of the noradrenaline that leaks from the storage vesicles; b) low passive diffusion of the amine across the axonal membrane, and c) little carried-mediat-

ed outward transport of noradrenaline. However, the latter mechanism is greater in the rat atrium than in other tissues (Schömig et al. 1989) and it probably accounts for the higher proportion of unchanged [^{3}H]-noradrenaline we found during efflux.

According to Sammet and Graefe (1979) and Stute and Trendelenburg (1984) the carrier-mediated outward transport of noradrenaline is increased whenever the axoplasmic Na$^+$ concentration increases. The rise in cytoplasmic Na$^+$ concentration induces an acceleration of noradrenaline efflux (Paton 1973), partly because the availability of the carrier at the inside of the axonal membrane increases and also because the affinity of noradrenaline for the inside carrier increases (Sammet and Graefe 1979). The activation of fast Na$^+$ channels and an increase in Na$^+$ influx induced by Bay K 8644 have been reported recently (Kohlhardt et al. 1989; Yatani et al. 1988). However, if this mechanism were to account for the Bay K 8644-induced increase in the efflux of tritium, a different metabolic pattern of tritiated compounds should be expected. Any time that axoplasmic Na$^+$ concentration increases, the efflux of noradrenaline exceeds the one of DOPEG and thus the ratio noradrenaline/DOPEG increases (Stute and Trendelenburg 1984). This was not the case. In addition, cocaine does not prevent the chronotropic action of Bay K 8644 (Camilión de Hurtado et al. 1989) indicating that uptake$_1$ is not involved in the effect of Bay K 8644.

The metabolic profile of Bay K 8644-induced overflow of tritium resembles the metabolic profile of reserpine-induced overflow of tritium. Reserpine and the reserpine-like agent, Ro 4-1284, increase FRL of tritium with a selective increase in the proportion of [^{3}H]-DOPEG and a consequent decrease in the ratio [^{3}H]-noradrenaline/[^{3}H]-DOPEG (Adler-Graschinsky et al. 1972; Stute and Trendelenburg 1984). Both drugs also produce positive chronotropic effect on isolated atria (Brimijoin and Trendelenburg 1971; Pesce and Adler-Graschinsky 1983). The extent of changes in the FRL of tritium and its metabolic distribution induced by 3 µmol/l Bay K 8644 is comparable to the effect of 0.1 µmol/l reserpine (Stute and Trendelenburg 1984). Also the moderate increase in atrial rate induced by reserpine (Brimijoin and Trendelenburg 1971) and the chronotropic effect of Bay K 8644 reported here are comparable.

However, unlike reserpine, the effect of Bay K 8644 on the spontaneous efflux of tritium was Ca^{2+}-dependent as shown by the results of Fig. 2, panel C. This calcium dependency was also shown in the study of Mundiña et al. (1989) who reported that the postsynaptic β-receptor-mediated effects of Bay K 8644 (i.e. increases in myocardial cAMP levels and relaxation) were suppressed when extracellular Ca^{2+} was lowered.

Two speculative alternatives can be forwarded in order to explain the calcium dependency for the Bay K 8644-induced overflow of tritium. The first of the possibilities should be that Bay K 8644, as other dihydropyridines do (Glossmann et al. 1982), requires calcium for binding to its receptor sites. Secondly, the releasing effect of Bay K 8644 might be the result of an indirect effect of increased axoplasmic Ca^{2+} concentration. It has been shown that the increase of Ca^{2+} could decrease intracellular pH (Ahmed and Connor 1980; Meech and Thomas 1977; Mullins et al. 1983) which in turn might interfere with the normal proton gradient of the storage vesicles (Beers et al. 1982; Phillips 1982; Winkler et al. 1986).

A puzzling finding was that, despite its calcium dependency, the effect of Bay K 8644 on tritium efflux could not be inhibited by nitrendipine. This could be interpreted as indicating that the L-subtype voltage-dependent calcium channel is not involved in the increase in tritium efflux reported here. This contention is further supported by the lack of effect of Bay K 8644 on the depolarization-induced overflow of tritium.

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