A novel mechanism for the melatonin inhibition of testosterone secretion by rat Leydig cells: reduction of GnRH-induced increase in cytosolic Ca^{2+}

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ABSTRACT

The site of inhibition, by melatonin, of GnRH-dependent testosterone secretion was investigated in adult rat Leydig cells cultured *in vitro*. The various effects downstream of the binding of GnRH to its own receptor were isolated and mimicked by specific drugs. Testosterone secretion was then evaluated after 3 h stimulation with GnRH, thapsigargin (1 μM), phorbol-12-myristate-13-acetate (100 nM), arachidonic acid (20 μM), and ionomycin (1 μM) in the presence or absence of melatonin (215 nM). The effect of melatonin on the GnRH-induced changes in cytoplasmic calcium concentration ($[\text{Ca}^{2+}]_i$) was also studied, using Fura-2 as fluorescent Ca^{2+} indicator. Melatonin attenuated the increase in $[\text{Ca}^{2+}]_i$ and inhibited the testosterone secretion induced by GnRH, but not that induced by ionomycin. Both ionomycin and thapsigargin potentiated GnRH-induced testosterone secretion; however, ionomycin, but not

thapsigargin, partially prevented the inhibitory effect of melatonin on cells stimulated with GnRH. The effect of melatonin was probably dependent on the binding of melatonin to its Gi-protein-coupled receptor, as the inhibitory effect on GnRH-induced secretion was suppressed in cells pretreated with pertussis toxin in a concentration of 180 ng/ml for 20 h. Assay of 17-hydroxy-progesterone showed that, irrespective of the treatment, cells cultured with melatonin secreted greater amounts than controls. We conclude that melatonin reduces GnRH-induced testosterone secretion by 1) decreasing $[\text{Ca}^{2+}]_i$, through impairment of the GnRH-dependent release of Ca^{2+} from intracellular stores and 2) blocking 17–20 desmolase enzymatic activity, an effect that occurs irrespective of changes in $[\text{Ca}^{2+}]_i$.

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INTRODUCTION

Testosterone production by Leydig cells is stimulated not only by luteinizing hormone (LH), which activates the cAMP pathway, but also by several endocrine and paracrine factors, which can act through non-cAMP-dependent pathways (Saez 1994). Among these, gonadotropin-releasing hormone (GnRH) exerts its action through a complex interplay of signalling pathways, the final results being an increase in cytosolic Ca^{2+} concentrations and activation of protein kinase C (PKC) – probably more relevant for the stimu-

lation of testosterone secretion. After the binding of GnRH to its own receptor, inositol triphosphate (IP_3) and diacylglycerol (DAG) are produced through activation of phospholipase C (PLC). IP_3 releases calcium from the intracellular stores, and DAG directly regulates the activity of PKC (Leung & Steele 1992, Rasmussen *et al.* 1995). Some DAG may be hydrolysed by DAG lipase to produce arachidonic acid; generation of the latter also occurs after activation of phospholipase A2 (PLA_2) by GnRH, and can directly stimulate androgen secretion (Lin 1985, Romanelli *et al.* 1995).

Melatonin has been shown to modulate directly the *in vitro* secretion of testosterone by rat (Ellis 1972, Ng & Lo 1988, Valenti *et al.* 1995) and human Leydig cells (Giusti *et al.* 1997). Indeed, after binding to its pertussis-toxin-sensitive receptor, melatonin reduces LH-stimulated testosterone secretion by inhibiting adenyl cyclase activity (Valenti *et al.* 1997). However, melatonin is also likely to influence non-cAMP mediated testosterone secretion, as it reduces GnRH-dependent testosterone secretion also (Valenti *et al.* 1995, 1997). A comparable effect of melatonin has been noted by Vanecek (1998) in the control of GnRH-induced LH secretion from neonatal rat gonadotrophs; in these cells, melatonin inhibits the GnRH-induced increase in Ca^{2+} by interfering both with the mobilization of Ca^{2+} from intracellular stores and with the subsequent entry of extracellular Ca^{2+} .

In the present study, the mechanism(s) by which melatonin affects GnRH-mediated testosterone secretion has therefore been investigated. To this end, the effects evoked by GnRH downstream of the binding to its receptor were separated into several components, which were mimicked by specific drugs. Subsequently, the effect of melatonin was tested on the secretion of testosterone induced by the naturally occurring sesquiterpene lactone, thapsigargin, which increases intracellular calcium concentrations (Putney & Bird 1993), phorbol myristate acetate (PMA), which directly activates PKC (Foresta *et al.* 1995), the ionophore, ionomycin, which allows extracellular calcium entry (Pereira *et al.* 1988), and direct administration of arachidonic acid, which directly stimulates testosterone secretion (Lin 1985, Romanelli *et al.* 1995). Moreover, the effect of melatonin on the GnRH-induced changes in cytoplasmic Ca^{2+} concentrations was studied by using the fluorescent Ca^{2+} indicator, Fura-2.

MATERIALS AND METHODS

Chemicals

Collagenase (type IA), soybean trypsin inhibitor, leupeptin, BSA (fraction V), PMA, arachidonic acid, thapsigargin, ionomycin, and pertussis toxin (PTX) were purchased from Sigma Chemical Co. (St Louis, MO, USA); melatonin was obtained from Bachem (Torrance, CA, USA); human LH was obtained from the National Institute for Biological Standards and Control (Potters Bar, UK); GnRH was purchased from Serono (Rome, Italy). Percoll was obtained from Pharmacia (Uppsala, Sweden); medium 199 and Hanks' balanced salt solution (HBSS) were obtained from

Gibco (Grand Island, NY, USA). Fura-2 acetoxymethyl ester (Fura-2) was purchased from Calbiochem (La Jolla, CA, USA).

Leydig cell purification

Adult male Wistar rats (70 days old), obtained from Morini (S Polo d'Enza, Italy) were maintained on a 12 h light : 12 h darkness cycle (lights on 0600–1800 h). All experiments were performed soon after the death of the rat, which took place at 0900 h. Leydig cell purification was performed as previously described (Conte *et al.* 1993). Immediately after death, the testes were removed and infused, via the testicular artery, with an enzyme solution (collagenase 0.5 mg/ml, soybean trypsin inhibitor 0.2 mg/ml, and leupeptin 5 µg/ml in HBSS containing 0.1% (wt/vol) BSA, pH 7.4) and then dispersed in enzyme solution in a shaking waterbath (20 min; 90 cycles/min; 34 °C). After the dissociated tubules had been allowed to settle, the supernatants were filtered, washed and centrifuged. The pellet was resuspended, applied to a discontinuous Percoll density gradient and centrifuged. Leydig cells that migrated to the 43–68% interface (specific gravity 1.064–1.096 g/ml) were collected, resuspended in medium 199 and centrifuged once more. The percentage of Leydig cells obtained was $78 \pm 6\%$, as determined by histochemical staining for 3β -hydroxysteroid dehydrogenase, and cell viability was $>90\%$, as determined by trypan blue exclusion. Cells were counted in haemocytometer, aliquoted in 96-well plates ($4 \times 10^4/0.2$ ml medium 199 per well), and allowed to attach during 1 h of preincubation at 37 °C in an atmosphere of 95% O_2 and 5% CO_2 .

Cell cultures

Cells were incubated for 3 h with increasing doses of the drugs to be tested. One optimal dose was selected for each substance and used in further experiments, which were conducted in the presence or absence of melatonin 215 nM (50 ng/ml). Thapsigargin and ionomycin were added 15 min before administration of GnRH, melatonin, or both. To test whether the effect exerted by melatonin was mediated by the binding of the indole to its own membrane receptor coupled to a PTX-sensitive Gi protein, some experiments were repeated with cells pretreated or not with PTX (180 ng/ml for 20 h). PMA was used as a factor able to induce secretion of testosterone through direct activation of PKC. To confirm the involvement of PKC, some experiments were performed in the presence of the PKC inhibitor, staurosporine (30 nM, administered 30 min before the stimuli) in basal conditions and

during stimulation with GnRH, PMA and LH (20 mIU/ml). Finally, the role of melatonin on arachidonic acid-dependent testosterone secretion was evaluated, by administering the drug in a dose of 20 μ M.

Cytoplasmic Ca^{2+} measurements

Leydig cells were seeded on coverslips placed in individual Petri dishes (2 ml/dish) and allowed to attach for 60 min at 37 °C. After a 10-min wash, cells were loaded with 4 μ M Fura-2 for 30 min at room temperature to avoid probe compartmentalization (Malgaroli *et al.* 1987), and then washed again for 10 min. Coverslips were mounted on a coverslip chamber for fluorescence measurement, which was performed at room temperature in medium 199, on individual fields of 25–30 cells each. To test the effects of melatonin, the hormone was added to the medium 199 in a dose of 10 μ M during the loading period and throughout the experiment. Each cell in the image was independently analysed for each timepoint in the captured sequence. For the calibration of fluorescent signals, cells loaded with Fura-2 were used. R_{max} and R_{min} are ratios at saturating and zero $[\text{Ca}^{2+}]_i$ respectively, and were obtained by perfusing cells with standard buffer containing 10 mM CaCl_2 , 5 μ M ionomycin and, subsequently, a Ca^{2+} -free solution containing 20 mM EGTA and 40 mM Tris. The values of R_{max} and R_{min} , expressed as a grey-level mean, were used to calculate the calibration curve by mean of TARDIS software (Scorziello *et al.* 1997). $[\text{Ca}^{2+}]_i$ was determined according to the equation of Grynkiewicz *et al.* (1985).

RIAs

Testosterone and 17-hydroxyprogesterone (OHP) were assayed by RIA (Diagnostic Product Corp., Los Angeles, CA, USA) as previously reported (Valenti *et al.* 1997). The sensitivity level for testosterone was 0.52 nM/l and the intra-assay coefficient of variation (CV) was 5.3% at 1.42 nM/l; for OHP, sensitivity was 0.3 nM/l and the CV was 7.8% at 2.4 nM/l. None of the substances tested interfered with the assays.

Statistics

Data from at least three experiments were pooled, analysed by ANOVA followed by unpaired Student's *t*-test and expressed as mean $\pm$ S.E.M. For comparison of multiple treatment groups with a single control group, Dunnett's *post hoc* test was applied. Significance was taken as $P < 0.05$.

FIGURE 1. Testosterone secretion recorded after 3 h of incubation in basal conditions and during stimulation with GnRH, thapsigargin and ionomycin, by control cells and cells cultured in the presence of melatonin (MLT) (215 nM). Experiments were performed in the presence (upper panel) and in the absence (lower panel) of extracellular Ca^{2+} . Significant differences from $^{\circ}$ basal and * control cells ($P < 0.05$).

RESULTS

Effect of melatonin on testosterone release stimulated by GnRH and Ca^{2+} mobilizing stimuli

A dose-dependent increase in testosterone secretion was noted during incubation with increasing concentrations of GnRH (1 nM–1 μ M), thapsigargin (0.01–1 μ M) and ionomycin (0.01–1 μ M).

The concentrations selected for the present experiments were 100 nM for GnRH and 1 μ M for thapsigargin and ionomycin; in the presence of melatonin (215 nM), the testosterone secretion evoked by GnRH and thapsigargin was significantly reduced, whereas melatonin did not affect the testosterone secretion elicited by administration of ionomycin. The results of these experiments are shown in Fig. 1, upper panel. In Ca^{2+} -free medium, GnRH and thapsigargin still increased testosterone secretion, although to a lower extent than that recorded in medium 199, and administration of melatonin suppressed this secretion. In contrast, ionomycin administered to cells cultured in

FIGURE 2. Upper panel: GnRH-induced testosterone secretion as recorded after 3 h of incubation with GnRH alone (Control) or with the addition of ionomycin (1 μ M), melatonin (MLT) (215 nM) or ionomycin+MLT. *Significant difference from controls ($P<0.05$). Lower panel: GnRH-induced testosterone secretion as recorded after 3 h of incubation with GnRH alone (Control) or with the addition of thapsigargin (1 μ M), MLT (215 nM) or thapsigargin+MLT. *Significant difference from controls ($P<0.05$).

Ca²⁺-free medium did not significantly influence testosterone secretion, and the effect of melatonin was noticeable. These results are shown in the lower panel of Fig. 1.

Ionomycin administered to cells bathed in medium 199 suppressed the inhibitory effect of melatonin on GnRH-dependent testosterone secretion; indeed, cells incubated with ionomycin+melatonin+GnRH secreted an amount of testosterone similar to that secreted by those cells treated with GnRH alone (Fig. 2, upper panel). The use of increasing concentrations of ionomycin did not reverse this effect; indeed, irrespective of the concentrations of the ionophore administered, testosterone secretion by cells incubated with GnRH and melatonin always lagged behind that of cells cultured with GnRH (data not shown). Furthermore, concentrations of ionomycin greater than 10 μ M resulted in impaired testosterone secretion, possibly because of a toxic effect of

FIGURE 3. The effect of administration of melatonin (MLT) on secretion of testosterone (upper panel) and cAMP (lower panel) by cells incubated in basal conditions or with GnRH and LH. Cells had been preincubated in the presence (PTX and PTX+MLT) or in the absence (Control and MLT) of PTX (180 ng/ml for 20 h) and washed before 3 h incubation in the presence (PTX+MLT and MLT) or in the absence (Control and PTX) of MLT (215 nM/l). Significant difference from basal and *Controls ($P<0.05$).

the drug at these concentrations. In contrast, thapsigargin was not able to prevent the inhibitory effect exerted by melatonin on GnRH-dependent testosterone secretion, either at the dose reported in the lower panel of Fig. 2, or in greater concentrations (data not shown).

To test whether the effect of melatonin was mediated by the binding of the indole to its own Gi-protein-coupled membrane receptor, some experiments were repeated after pretreatment with PTX in a concentration of 180 ng/ml for 20 h. As a result of PTX pretreatment, basal and LH-stimulated testosterone secretions were greater than those recorded in controls; furthermore, the inhibitory effect of melatonin on GnRH- and LH-stimulated secretion was eliminated. cAMP secretion was comparable to that recorded in controls, both in the basal condition and during GnRH-stimulation, but varied according to the changes in testosterone secretion during

FIGURE 4. Mean $[Ca^{2+}]_i$ as recorded over 360 s from 200 control cells ($\circ$) and 200 cells incubated with melatonin (MLT) ($1 \mu M$, $\bullet$). Acutely added GnRH ($10 \mu M$) evoked a rapid and transient $[Ca^{2+}]_i$ spike; in the presence of MLT, basal $[Ca^{2+}]_i$ and the GnRH-induced $[Ca^{2+}]_i$ spike were reduced. Insert: Basal $[Ca^{2+}]_i$ and GnRH-induced $[Ca^{2+}]_i$ peak in the absence or presence of increasing concentrations of MLT (0.1 – $10 \mu M$) (means of four experiments). Significant differences from the corresponding control incubated without MLT: * $P < 0.05$, ** $P < 0.01$.

administration of LH. These results are presented in Fig. 3.

Effect of melatonin on direct cytoplasmic Ca^{2+} measurement

Mean $[Ca^{2+}]_i$ concentrations recorded from five experiments for controls and melatonin-treated cells in response to GnRH administration are shown in Fig. 4. Addition of GnRH ($10 \mu M$) caused a rapid and transient increase in $[Ca^{2+}]_i$ in 38% of the cells observed; after the spike response, $[Ca^{2+}]_i$ returned to basal values within 60 s. Cells incubated in the presence of melatonin ($1 \mu M$) showed significantly lower basal $[Ca^{2+}]_i$ concentrations and a significant reduction in the peak amplitude after GnRH administration; however, melatonin administration did not influence the number of cells responsive to GnRH (39%) in comparison with the control group. Comparable results were noted in Ca^{2+} -free medium (data not shown).

The inhibitory effect of melatonin on the GnRH-dependent increase in intracellular Ca^{2+} was a dose-dependent phenomenon, and appeared for concentrations of melatonin greater than $0.1 \mu M$ (insert to Fig. 4).

Effects of melatonin on testosterone secretion dependent on direct PKC stimulation and exogenous arachidonic acid administration

PMA increased the testosterone secretion in a dose-dependent manner (0.01 – $10 \mu M$); the concentration of 100 nM was selected for these experiments. Administration of melatonin did not change testosterone secretion in the basal condition or in the presence of staurosporine, a blocker of PKC activity (data not shown); however, melatonin administration did reduce GnRH- and PMA-stimulated testosterone secretion. In the presence of staurosporine, PMA- and GnRH-dependent testosterone production were reduced by 30% and 40% respectively, whereas spontaneous or LH-stimulated (30 mIU/ml) testosterone secretions were unaffected by staurosporine. The results of these experiments are shown in Fig. 5.

Arachidonic acid administered in a concentration of $20 \mu M$ increased testosterone release by approximately 50%; melatonin impaired this secretion of testosterone (Fig. 6).

Effect of melatonin on 17-hydroxy-progesterone

OHP concentrations were recorded from the experiments described; they are summarized in

FIGURE 5. Secretion of testosterone recorded after 3 h of incubation in basal conditions and during stimulation with GnRH and PMA, by control cells and cells cultured in the presence of melatonin (MLT) (215 nM). Insert: Testosterone secretion recorded in basal conditions, in the presence of GnRH (100 nM), PMA (100 nM) and LH (20 mIU/ml) for control cells and cells preincubated with staurosporine (30 nM for 30 min). These results are expressed as percentage from control cells. *Significant difference from control cells ($P < 0.05$).

Table 1, together with testosterone concentrations. All the stimuli used increased OHP concentrations over the corresponding basal values, both in the presence and in the absence of melatonin. However, irrespective of the drug administered, OHP concentrations were always three- to fourfold greater in the media harvested from cells treated with melatonin than in their corresponding controls, and the secretion of testosterone was significantly reduced.

FIGURE 6. Testosterone secretion as recorded after 3 h of incubation in basal conditions and during stimulation with arachidonic acid in the presence or absence of melatonin (MLT) (215 nM). Significant differences from °basal and *Control cells ($P < 0.05$).

DISCUSSION

This study has shown that melatonin inhibits GnRH-dependent testosterone secretion, in part by decreasing the cytoplasmic Ca^{2+} concentrations in rat Leydig cells cultured *in vitro*. In our experiments, melatonin blocked the testosterone secretion induced by GnRH and thapsigargin, which release Ca^{2+} ions from intracellular stores, and this inhibitory effect of melatonin was also observed in the absence of extracellular calcium.

When administered to cells bathed in medium 199, the ionophore, ionomycin, was capable of reversing the inhibitory effect of melatonin on testosterone secretion. Ionomycin, by allowing transmembrane Ca^{2+} influx, replenishes the intracellular Ca^{2+} stores, which are likely to be depleted within a few minutes (Li *et al.* 1994, Foresta *et al.* 1996), and thus enables steroidogenesis to occur. Indeed, although the early response to GnRH in Leydig cells involves mobilization of Ca^{2+} from the intracellular stores without requiring extracellular Ca^{2+} influx (Tomic *et al.* 1995), steroidogenesis has been demonstrated to be highly dependent on the extracellular Ca^{2+} influx, as studies performed in Ca^{2+} -free medium or in the presence of verapamil, an antagonist of Ca^{2+} channels, have demonstrated (Sullivan & Cooke 1986, Foresta *et al.* 1996). Both ionomycin and thapsigargin augmented the

TABLE 1. Concentration of 17-hydroxy-progesterone (OHP) and testosterone (T) as determined after incubation of Leydig cells for 3 h in the presence of various stimuli

	Controls		MLT	
	OHP (nM/l)	T (nM/l)	OHP (nM/l)	T (nM/l)
Stimulus				
Basal	0.28 ± 0.05	8.65 ± 1.03	1.01 ± 0.15†	7.61 ± 0.92†
GnRH (100 nM)	0.55 ± 0.05*	20.20 ± 1.55*	2.44 ± 0.16*,†	12.05 ± 1.51*,†
PMA (100 nM)	0.34 ± 0.08	12.75 ± 1.13*	1.19 ± 0.20†	9.21 ± 0.98†
AA (20 µM)	0.36 ± 0.02*	10.01 ± 1.07*	1.25 ± 0.15*,†	7.77 ± 0.38†
Thapsigargin (1 µM)	0.76 ± 0.09*	24.22 ± 3.46*	2.10 ± 0.25*,†	9.51 ± 1.21†
Thapsigargin + GnRH	1.53 ± 0.30*	33.73 ± 4.32*	7.25 ± 1.25*,†	11.42 ± 3.46*,†
Ionomycin (1 µM)	0.86 ± 0.10*	14.01 ± 2.35*	2.67 ± 0.35*,†	12.87 ± 3.80*,†
Ionomycin + GnRH	1.65 ± 0.28*	25.95 ± 1.21*	6.07 ± 1.37*,†	17.64 ± 2.77*,†

AA, arachidonic acid. * $P < 0.05$ compared with basal; † $P < 0.05$ compared with Controls.

GnRH-dependent secretion of testosterone, but only ionomycin was capable of reversing the inhibitory effect of melatonin on such secretion. The inhibitory role of melatonin on cytosolic Ca^{2+} concentrations was also demonstrated by direct measurement of cytosolic Ca^{2+} concentrations by Fura-2. Approximately one-third of the Leydig cell population responded to high-dose administration of GnRH with an early spike in $[\text{Ca}^{2+}]_i$, which was not followed by a sustained plateau phase, even when bathed in Ca^{2+} -free medium. These results are in accordance with those reported by Tomic *et al.* (1995), who demonstrated for the first time that only a part of the Leydig cell population is responsive to GnRH. In our experiments, the GnRH-dependent $[\text{Ca}^{2+}]_i$ spike was reduced in a dose-dependent manner by melatonin, showing that the effect of melatonin in decreasing $[\text{Ca}^{2+}]_i$ is in part due to a reduction in the release of Ca^{2+} from intracellular stores.

It has previously been shown that the inhibitory effect of melatonin on GnRH-induced LH secretion by neonatal gonadotrophs is blocked by PTX pretreatment, suggesting the involvement of an inhibitory G-protein (Vanecek 1998). Similarly, in our cells, the inhibitory effect was suppressed by pretreatment with PTX in a concentration of 180 ng/ml for 20 h, thus suggesting the participation of the same kind of melatonin membrane receptor; indeed, Leydig cells bear melatonin receptors that are coupled to PTX-sensitive G-proteins (Valenti *et al.* 1997).

Although, in neonatal gonadotrophs, the negative effect of melatonin on GnRH-induced LH secretion was progressively eliminated by increasing concentrations of BAY K or A 23187 (Vanecek 1998), in our cells the inhibitory effect of melatonin on GnRH-dependent testosterone secretion was never completely reversed in any of the experiments with

ionomycin; these discrepancies can be explained on the basis that, in Leydig cells, other mechanism(s) are likely to be involved in the melatonin-dependent blockade of GnRH-induced steroidogenesis. Given the fact that melatonin had been shown to be able to decrease DAG formation in pituitary cells (Dix *et al.* 1984), we were expecting PMA administration somehow to prevent the inhibitory role of melatonin. This phenomenon was not observed in our experiments; indeed, testosterone secretion was reduced by melatonin in the presence of factors capable either of directly activating (PMA) or of inhibiting (staurosporine) PKC activity (Foresta *et al.* 1995, Vanecek 1998). Similarly, administration of increasing concentrations of PMA was not able to reverse the inhibitory effect of melatonin on GnRH-dependent LH secretion in neonatal rat gonadotrophs (Didolkar & Sundaram 1989).

The inhibitory effect of melatonin has also been observed on the testosterone secretion induced by arachidonic acid, which directly stimulates testosterone secretion independently of its cyclo-oxygenase and lipoxygenase products (Lin 1985, Romanelli *et al.* 1995).

Direct measurement of OHP concentrations confirmed that, in all the experiments, melatonin reduced the enzymatic activity along the steroidogenic pathway – an effect attributable to a block of 17–20 desmolase (Valenti *et al.* 199, 1997); in particular, none of the drugs administered, alone or in combination, was able to reverse this effect – not even high concentrations of ionomycin. These results suggest that the mechanism(s) implicated in the melatonin-dependent regulation of 17–20 desmolase enzymatic activity does not involve any of the mediators elicited downstream of the binding of GnRH to its receptor – not even changes in $[\text{Ca}^{2+}]_i$. Melatonin could, therefore, act through still unknown mechanism(s), somehow controlling the

expression of enzymes of the steroidogenic pathway, other proteins, or both.

We conclude that melatonin reduces GnRH-induced testosterone secretion by 1) decreasing $[Ca^{2+}]_i$, through impairment of the GnRH-dependent release of Ca^{2+} from intracellular stores, and 2) by blocking 17–20 desmolase enzymatic activity, an effect that occurs irrespective of changes in $[Ca^{2+}]_i$.

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