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Biochemical and Biophysical Research Communications 337 (2005) 104–109
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Influence of the degree of unsaturation of the acyl side chain upon the interaction of analogues of 1-arachidonoylglycerol with monoacylglycerol lipase and fatty acid amide hydrolase ☆

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Received 18 August 2005
Available online 13 September 2005

Abstract

Little is known as to the structural requirements of the acyl side chain for interaction of acylglycerols with monoacylglycerol lipase (MAGL), the enzyme chiefly responsible for the metabolism of the endocannabinoid 2-arachidonoylglycerol (2-AG) in the brain. In the present study, a series of twelve analogues of 1-AG (the more stable regioisomer of 2-AG) were investigated with respect to their ability to inhibit the metabolism of 2-oleoylglycerol by cytosolic and membrane-bound MAGL. In addition, the ability of the compounds to inhibit the hydrolysis of anandamide by fatty acid amide hydrolase (FAAH) was investigated. For cytosolic MAGL, compounds with 20 carbon atoms in the acyl chain and 2–5 unsaturated bonds inhibited the hydrolysis of 2-oleoylglycerol with similar potencies (IC_{50} values in the range 5.1–8.2 μM), whereas the two compounds with a single unsaturated bond were less potent (IC_{50} values 19 and 21 μM). The fully saturated analogue 1-monoarachidin did not inhibit the enzyme, whereas the lower side chain analogues 1-monopalmitin and 1-monomyristin inhibited the enzyme with IC_{50} values of 12 and 32 μM, respectively. The 22-carbon chain analogue of 1-AG was also potent (IC_{50} value 4.5 μM). Introduction of an α-methyl group for the C20:4, C20:3, and C22:4 compounds did not affect potency in a consistent manner. For the FAAH and the membrane-bound MAGL, there was no obvious relationship between the degree of unsaturation of the acyl side chain and the ability to inhibit the enzymes. It is concluded that increasing the number of unsaturated bonds on the acyl side chain of 1-AG from 1 to 5 has little effect on the affinity of acylglycerols for cytosolic MAGL.

Keywords: 2-Arachidonoylglycerol; Anandamide; Monoacylglycerol lipase; Fatty acid amide hydrolase

The endocannabinoid system has been shown to be involved in a number of important physiological processes, including control of emotional states, appetite, and pain perception [1–3]. In the brain, considerable interest has been devoted to the abilities of endocannabinoids to act as retrograde signalling molecules regulating the release of transmitters such as glutamate and GABA [4]. The two most well-studied endocannabinoids are anandamide (AEA, *N*-arachidonoylethanolamine) and 2-arachidonoylglycerol (2-AG) [5–7]. These two molecules have short durations of action, due to effective routes of metabolism. The two main enzymes involved are fatty acid amide hydrolase (FAAH, for both AEA and 2-AG) [8,9] and monoacylglycerol lipase (MAGL, for 2-AG) [10]. In the brain, MAGL is primarily responsible for the metabolism

☆ *Abbreviations:* AEA, anandamide, *N*-arachidonoylethanolamine; 1-AG, 1-arachidonoylglycerol; 2-AG, 2-arachidonoylglycerol; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; 2-OG, 2-oleoylglycerol; URB597, 3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate.

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doi:10.1016/j.bbrc.2005.09.015

of 2-AG [10–12] and there is evidence to suggest that inhibition of this enzyme can potentiate retrograde signalling in hippocampal pyramidal cells [13].

FAAH, a ~63 kDa membrane-bound enzyme [14] which is localised postsynaptically in hippocampus and cerebellum [15], has a wide substrate specificity and can metabolise acyl-ethanolamines and acyl-amides with both fully saturated as well as unsaturated side chains [16–18]. In contrast, less is known about the substrate specificity of MAGL, a 33 kDa enzyme which is found both in cytosolic and membrane fractions [10,11,19] and which is localised presynaptically in the hippocampus and cerebellum [13]. It is, however, clear that MAGL overexpressed in HeLa cells is capable of metabolising 2-oleoylglycerol (2-OG) and 2-AG, but not AEA [10] and that the 1(3)-regioisomer of 2-OG is metabolised as effectively as 2-OG itself [19]. A similar result has been reported for 1(3)- and 2-AG for membrane-bound MAGL from rat cerebellum [11].

Whilst it is clear that MAGL can metabolise oleoyl- and arachidonoyl-glycerols, its ability to interact with other acyl glycerols is less clear. An early report that rat liver microsomal particulate fractions could metabolise 1-palmitoylglycerol and 1-OG with about the same activities (and less than the activity towards 1-myristoylglycerol as substrate) may, however, reflect the ability of FAAH to metabolise these substrates, since the observed molecular weight of the purified enzyme was 62–63 kDa [20]. A study using rabbit aorta as enzyme source and showing similar rates of metabolism of 2-OG and 2-palmitoylglycerol [21] may also reflect an influence of FAAH, since the authors used a mixture of the cytosolic and detergent-solubilised enzymes.

One simple way of investigating the interaction between MAGL and acyl compounds is to determine their ability to prevent the enzyme from hydrolysing [^{3}H]2-OG. Although this does not give information as to the efficacy of the compounds as substrates, it does allow determination of their affinity for the enzyme. Using such an approach, we have recently reported that 2-lineleoylglycerol (C18:2, where the numbers refer to the length of the acyl side chain and the number of double bonds, respectively) inhibited [^{3}H]2-OG hydrolysis by cytosolic MAGL with a similar potency to 2-AG (C20:4) and non-radioactive 2-OG (C18:1) itself [22]. In the present study, we have extended these data to a series of compounds with different degrees of unsaturation with respect to their ability to interact with both cytosolic and membrane-bound MAGL and, for comparative purposes, with FAAH . The compounds are based upon 1-AG rather than 2-AG in view of the lack of stability of the latter in biological solutions [23].

Materials and methods

Compounds. Radiolabelled 2-mono-oleoylglycerol [glycerol-1,2,3-^{3}H] ([^{3}H]2-OG, 20 Ci mmol^{-1}) and arachidonoylethanolamide [ethanolamine 1-^{3}H] ([^{3}H]AEA, 60 Ci mmol^{-1}) were obtained from American Radiolabeled Chemicals, St. Louis, MO, USA. α-Me-1-AG (α-methyl-1-arachidonoyl glycerol, compound O-1428), compounds O-3832, O-3846, O-3872, O-3907, O-3908, O-3973, O-4066, and O-4081 were synthesised in the laboratory of co-author Razdan using previously published procedures [24–26]. 1-Arachidinoylglycerol (1-monoarachidin), 1-palmitoylglycerol (1-monopalmitin), and 1-myristoylglycerol (1-monomyristin) were obtained from Research Plus, Manasquan, NJ, USA. 1-Arachidonoylglycerol (1-AG) and URB597 (3′-carbamoyl-biphenyl-3-yl-cyclohexylcarbamate) were obtained from the Cayman Chemical company, Ann Arbor, MI, USA. The structures of the acylglycerol compounds are shown in the table to aid the reader. 1-AG was dissolved in acetonitrile, whereas the other compounds were dissolved in ethanol, and solvent carrier concentrations were kept constant throughout the assays.

Assay of FAAH and MAGL. Cerebella previously obtained from adult Sprague–Dawley rats were thawed and homogenised at 4 °C in sodium phosphate buffer (50 mM, pH 8) containing 0.32 M sucrose. The homogenates were centrifuged at 100,000*g* for 60 min at 4 °C, and the supernatants (“cytosolic fractions”) were collected. The pellets were suspended in sodium phosphate buffer (50 mM, pH 8) (“membrane fractions”). The fractions were stored frozen in aliquots at −70 °C until used for assay. Protein concentrations of the fractions were determined [27] with bovine serum albumin as standard.

The assays for MAGL and FAAH have been described previously [10,22,28] (note that in [22], the concentration of fatty acid-free BSA used was inadvertently omitted). Briefly, membrane and cytosolic preparations were diluted to the appropriate assay protein concentrations (2 and 1.5 μg per assay for membrane and cytosolic fractions, respectively) in Tris–HCl buffer (10 mM, pH 7.2) containing 1 mM EDTA. Aliquots (165 μl) were then added to glass tubes containing 10 μl of test compound. Blanks contained assay buffer in place of membrane or cytosolic samples. [^{3}H]AEA (for FAAH) and [^{3}H]2-OG (for MAGL) (25 μl, final concentration 2 μM) were then added and the samples were incubated for 10 min at 37 °C. The substrate solution contained fatty acid-free BSA (final assay concentration 0.125% w/v). For the experiments using membrane fractions and [^{3}H]2-OG, the samples were coincubated with 3 μM URB597. This compound inhibits the FAAH activity without affecting MAGL [29], thereby allowing the measurement of membrane MAGL (see [28]). After the incubation phase, reactions were stopped by adding 400 μl chloroform/methanol (1:1 v/v), followed by vortex mixing the tubes twice and placing them on ice. Phases were separated by centrifugation for 10 min at 2500 rpm), and aliquots (200 μl) of the methanol/buffer phase containing the water soluble reaction products ([^{3}H]ethanolamine and [^{3}H]glycerol for FAAH and MAGL assays, respectively) were measured for tritium content by liquid scintillation spectroscopy with quench correction.

Analysis of data. The pooled data expressed as percentage of control activity containing the same carrier concentration were analysed using the built-in equation “sigmoidal dose–response (variable slope)” of the GraphPad Prism computer program (GraphPad Software, San Diego, CA, USA) as described previously [22].

Results

Interaction of analogues and homologues of 1-AG with MAGL and FAAH

Experiments were undertaken using nine concentrations of test compound in the range of 0.2–100 μM. As an example, the inhibition curves for the C20:3 analogue of 1-AG, compound O-3846, are shown in Fig. 1. The results for the compounds are shown in Table 1. For cytosolic MAGL, compounds with 20 carbon atoms in the acyl chain and 2–5 unsaturated bonds inhibited the hydrolysis of 2-oleoylglycerol with similar potencies (IC_{50} values in the range 5.1–8.2 μM), whereas the two compounds with a single

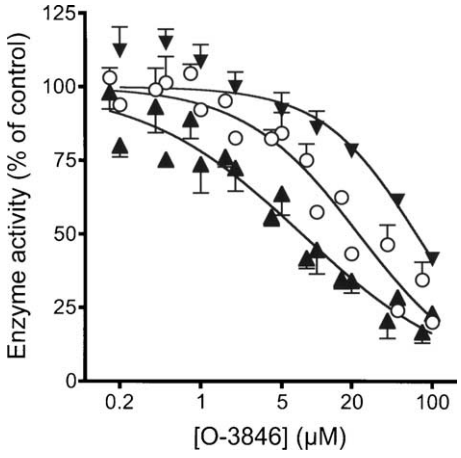


Fig. 1. Inhibition of the metabolism of [^{3}H]AEA by membrane-bound FAAH (○) and of [^{3}H]2-OG by cytosolic (▲) and membrane-bound (▼) MAGL. Shown are means ± SEM (when not enclosed by the symbols), where $n = 6$ (for FAAH and cytosolic MAGL) or $n = 3$ (for membrane-bound MAGL). The membrane-bound MAGL activity was measured in the presence of 3 μM URB597 to block FAAH-catalysed metabolism of [^{3}H]2-OG. The substrate concentration was 2 μM in all cases.

unsaturated bond were less potent (IC_{50} values 19 and 21 μM). The unsaturated compound (1-monoarachidin) was without effect upon the enzyme activity. However, the shorter chain homologues 1-monopalmitin (C16:0) and 1-monomyristin (C14:0) both inhibited the hydrolysis of 2-oleoylglycerol with IC_{50} values of 12 and 32 μM, respectively. Introduction of an α-methyl group for the C20:4 compound reduced the potency slightly, whereas the corresponding introduction into the C20:3 compound increased it slightly.

For the FAAH and the membrane-bound MAGL, there was no obvious relationship between the degree of unsaturation of the acyl side chain and the ability to inhibit the enzymes. Thus, for FAAH, 1-AG inhibited the activity with an IC_{50} value of 6.2 μM, and the 1–5 unsaturated bond analogues showed roughly similar potencies, with IC_{50} values ranging from 5.7 to 11 μM, with the exception of compound O-3846 (C20:3), which had an IC_{50} value of 23 μM. An almost 10-fold increase in potency was seen following introduction of the α-methyl group in the acyl side chain of this compound (compound O-3973, IC_{50} value 2.4 μM), although this was not seen in this series for the α-methyl analogue of 1-AG, which inhibited FAAH poorly. It should be noted that in these experiments, the compound was dissolved in ethanol. In a previous study, the compound was dissolved in acetonitrile and inhibited FAAH with an IC_{50} value of 33 μM [22]. When we rechecked this compound dissolved in acetonitrile, we obtained IC_{50} values towards FAAH and cytosolic MAGL of 69 and 44 μM, respectively (data not shown). The saturated 20:0 compound did not inhibit FAAH activity, whereas the smaller chain homologues 1-monopalmitin and 1-monomyristin inhibited the activity with IC_{50} values of 8 and 18 μM, respectively.

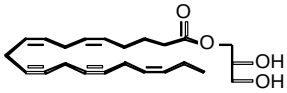
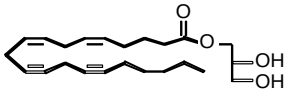
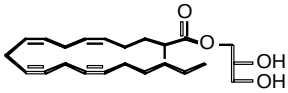
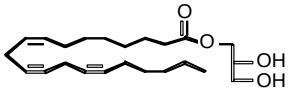
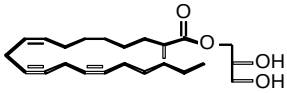
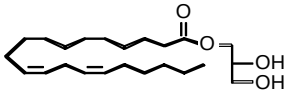
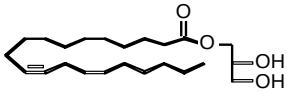
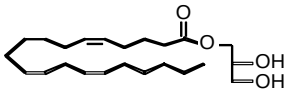
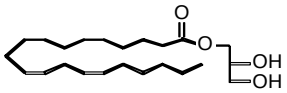
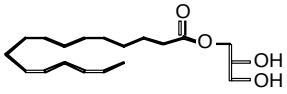
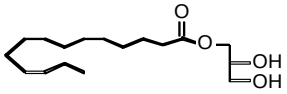
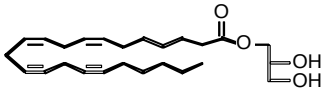
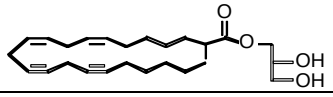
For membrane-bound MAGL, IC_{50} values for the unsaturated compounds ranged from 9.5 (1-AG) to 73 μM (compound O-3846). The saturated C14:0 homologue inhibited the MAGL with an IC_{50} value of 32 μM, whereas the C16:0 and C20:0 compounds were less efficacious. Introduction of the α-methyl group in the acyl side chain of the C20:3 compound slightly increased its potency, whereas a decreased potency was seen for the C20:4 analogue.

Two higher homologues of 1-AG were also tested. The C22:4 homologue (compound O-3872) had essentially the same affinity towards the three enzyme activities as 1-AG itself. Introduction of an α-methyl group in the side chain (compound O-4081) increased the inhibitory potency towards FAAH and membrane-bound MAGL, but not towards cytosolic MAGL.

Discussion

In the present study, the ability of a series of 1-acylglycerols to interact with MAGL and FAAH has been investigated. Before discussing the results, two important caveats should be mentioned. As pointed out in the introduction, the approach used allows determination of affinity towards the compounds, but not their efficacy as substrates, a situation analogous to the widely used radioligand binding technique in receptor research. Thus, for example, the inhibition of MAGL-catalysed [^{3}H]2-OG metabolism by 1-AG can be attributed to the compound acting as a competing substrate, whereas this is not necessarily true for the other compounds. This has been seen previously, where AEA inhibits [^{3}H]2-OG metabolism by cytosolic fractions with an IC_{50} value of 60 μM [22], but is not metabolised by MAGL [12]. An action as a competing MAGL substrate may also affect the potency towards FAAH, since such substrate efficacy would reduce the free concentration of the compound and hence its potency towards FAAH. This can neatly be exemplified by two observations: (a) Whilst the potency of 1-AG as inhibitor of cytosolic [^{3}H]2-OG metabolism seen here (IC_{50} value 7.1 μM) is in good agreement with our two previous studies investigating this compound (IC_{50} values 17 μM [22] and 7 μM) [30], a larger variation in the potency towards [^{3}H]AEA by the membrane-bound FAAH was seen in the three studies. The IC_{50} value here (7.6 μM) using 2 μg protein per assay was in good agreement with the previous data using this protein concentration (14 μM [30]) whereas in our original study using 4 μg protein per assay, complete inhibition was not seen at the highest concentration tested (100 μM) [22]. (b) It is well established that the introduction of an α-methyl group in the AEA side chain renders it more resistant to hydrolysis [17], and in our initial study, we found that α-methyl-1-AG inhibited FAAH much more potently than 1-AG, with little change in its ability to inhibit soluble MAGL [22]. A similar result was seen here for the α-methyl analogues of the C20:3 and C22:4 acylglycerols, although in this series of experiments, the C20:4

Table 1
Potencies of a series of analogues of 1-AG to inhibit the activity of FAAH and MAGL

Compound	Structure	pI_{50} (maximum inhibition attained) [IC_{50}]		
		FAAH	MAGL-m	MAGL-cy
O-3832 (C20:5)		5.12 ± 0.03 (100%) [7.6 μM]	4.77 ± 0.04 (100%) [17 μM]	5.08 ± 0.03 (100%) [8.2 μM]
1-AG (C20:4)		5.21 ± 0.04 (100%) [6.2 μM]	5.02 ± 0.03 (100%) [9.5 μM]	5.15 ± 0.10 (100%) [7.1 μM]
O-1428 (C20:4) (α-methyl)		<4 [28 ± 3% inhibition at 100 μM]	4.15 ± 0.09 (100%) [71 μM]	4.82 ± 0.05 (100%) [15 μM]
O-3846 (C20:3)		4.64 ± 0.04 (100%) [23 μM]	4.14 ± 0.07 (100%) [73 μM]	5.13 ± 0.05 (100%) [7.5 μM]
O-3973 (C20:3) (α-methyl)		5.61 ± 0.04 (95 ± 2%) [2.4 μM]	5.08 ± 0.05 (100%) [8.3 μM]	5.38 ± 0.11 (86 ± 7%) [4.2 μM]
O-3907 (C20:2)		5.25 ± 0.03 (100%) [5.7 μM]	4.81 ± 0.06 (100%) [16 μM]	5.29 ± 0.04 (100%) [5.1 μM]
O-4066 (C20:1)		4.99 ± 0.04 (100%) [10 μM]	4.59 ± 0.04 (100%) [26 μM]	4.72 ± 0.03 (79 ± 3%) [19 μM]
O-3908 (C20:1)		4.94 ± 0.10 (84 ± 6%) [11 μM]	4.25 ± 0.06 (100%) [56 μM]	4.68 ± 0.11 (65 ± 9%) [21 μM]
1-Mono-arachidin (C20:0)		<4 [10 ± 3% inhibition at 100 μM]	<4 [11 ± 7% inhibition at 100 μM]	<4 [19 ± 3% inhibition at 100 μM]
1-Mono-palmitin (C16:0)		5.10 ± 0.04 (100%) [8.0 μM]	<4 [44 ± 7% inhibition at 100 μM]	4.89 ± 0.09 (38 ± 4%) [12 μM]
1-Mono-myristin (C14:0)		4.73 ± 0.03 (100%) [18 μM]	4.50 ± 0.06 (100%) [32 μM]	4.49 ± 0.03 (100%) [32 μM]
O-3872 (C22:4)		4.87 ± 0.03 (100%) [14 μM]	4.98 ± 0.02 (100%) [11 μM]	5.35 ± 0.05 (100%) [4.5 μM]
O-4081 (C22:4) (α-methyl)		5.29 ± 0.06 (90 ± 4%) [5.1 μM]	5.07 ± 0.03 (100%) [8.5 μM]	5.24 ± 0.05 (100%) [5.8 μM]

Inhibition of membrane-bound FAAH, membrane-bound (m), and cytosolic (cy) MAGL by analogues of 1-AG. Data are means ± SEM of analyses of data from three to six experiments, using nine concentrations of test compound in the range of 0.2–100 μM. The computer program used to analyse the data returned pI_{50} values ($-\log_{10}IC_{50}$ for the inhibitable component) and the minimum remaining activity in the presence of inhibitor (i.e., 100-maximum attainable inhibition). A maximum inhibition <100% means that: (a) the 95% confidence limits of the minimum percentage values were both positive and (b) comparison of a model with a floating minimum baseline activity fitted the data significantly better than a model with 100% inhibition (for details, see [22]).

analogue did not follow the pattern. Ideally, the FAAH assays should be run in the presence of a potent and highly selective MAGL inhibitor (analogous to the use here of URB597 in the membrane-bound MAGL assays), but such a compound is not to our knowledge yet commercially available.

Despite the caveats discussed above, the present data provide much needed information upon the structural requirements of the acyl side chain of 1-AG for its interaction with MAGL and allow a number of conclusions to be made: (a) the number of unsaturated bonds in the acyl side chain is of minor importance for the interaction with the

soluble enzyme. Increasing the number of bonds from 2 (O-3907) to 5 (O-3832) does not change the potency for inhibition of [^{3}H]2-OG by the cytosolic fractions, whereas the situation for membrane-bound [^{3}H]2-OG metabolism is less clear. A loss in potency was seen when the monosaturated compounds were used, but the compounds retained activity. This is in contrast to the total loss of activity seen when cyclooxygenated derivatives of C20:4 acyl compounds are investigated [30] (a situation also seen for the interaction with FAAH [30,31].

For saturated compounds, previous data have shown that neither palmitoylethanolamide nor palmitoylserinol interacts with soluble MAGL to any significant extent, in contrast to their arachidonoyl equivalents [22]. However, palmitoyltrifluoromethyl ketone inhibits cytosolic [^{3}H]2-OG hydrolysis with an IC_{50} of 7.8 μM, which, although 100-fold less potent than its ability to inhibit FAAH, does indicate that saturated acyl compounds can interact with cytosolic MAGL [22]. Indeed, both 1-monomyristin (C14:0) and 1-monopalmitin (C16:0) inhibited MAGL (Table 1). The lack of effect of 1-monoarachidin (C20:0) may reflect a solubility issue, i.e., an inability to reach a critical concentration in the vicinity of the MAGL.

(b) α-Methylation of the acylglycerol does not consistently affect the observed affinity for cytosolic MAGL. The C20:3 (O-3973), C22:4 (O-4081), and C20:4 (O-1428) compounds inhibited cytosolic MAGL with IC_{50} values $\leqslant$15 μM. Although this inhibition is somewhat modest, particularly when compared to inhibitory potencies towards FAAH by potent inhibitors such as URB597 [29] and OL-135 (1-oxo-1[5-(2-pyridyl)-2-yl]-7-phenylheptane) [32], the potency is similar to that seen for inhibition of FAAH by compounds like phenylmethylsulphonyl fluoride [33] and arachidonoylserotonin [34], both of which have shown useful pharmacological activity in vivo [35,36]. Furthermore, the α-methyl analogue of 1-AG has only weak activity towards cannabinoid CB_1 receptors in vitro and does not produce overt signs of central cannabinoid receptor activation in vivo [22]. Given that 2-AG is metabolised by both FAAH and MAGL [9–11], and that whilst MAGL is predominant in the brain [10–12], FAAH may be more important in other regions of the body [see 37], a compound blocking both FAAH and MAGL may be useful to investigate the therapeutic potential of blockade of 2-AG metabolism in disease situations, such as trauma [38], where 2-AG levels are increased.

Acknowledgments

The research was also supported by grants from the Swedish Research Council (Grant No. 12158, medicine), Konung Gustav V's and Drottning Victorias Foundation, Gun and Bertil Stohne's Foundation, Stiftelsen för Gamla Tjänarinnor, and the Research Funds of the Medical Faculty, Umeå University. S.V. was supported by a post-doctoral fellowship from the foundation Wenner-Grenska Samfundet and an FSR (Fonds spécial de recherche) from the catholic university of Louvain. Financial support from the National Institute on Drug Abuse (NIDA Grant Nos. DA-09789 and DA-08904) is gratefully acknowledged (B.S., A.M., R.K.R., B.M. and R.G.P.).

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