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We are extremely saddened by the passing of Prof TSOU Kang, a Member of Chinese Academy of Sciences and the former Chairman of Department of Neuropharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. The following six articles are dedicated to the memory of Prof TSOU Kang.

Liquid chromatographic-mass spectrometric measurement of the endogenous cannabinoid 2-arachidonylglycerol in the spinal cord and peripheral nervous system

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KEY WORDS cannabinoids; 2-arachidonylglycerol; spinal ganglia; sciatic nerve; spinal cord; analgesia; peripheral nervous system; mass spectrum analysis

ABSTRACT

AIM: To develop a sensitive method for measuring the putative endocannabinoid 2-arachidonylglycerol (2-AG) in the peripheral and central nervous system. **METHODS**: A method using atmospheric pressure chemical ionization (APCI) liquid chromatography/ mass spectrometry (LC/MS) was developed to determine the levels of 2-AG in methanol extracts of the rat lumbar spinal cord, dorsal root ganglion (DRG), and sciatic nerve. **RESULTS**: 2-AG was detected with high sensitivity and minimal sample preparation. The levels in the tissues analyzed were ≤ pmol/mg wet weight. Similar levels were found in the spinal cord and the DRG, whereas approximately 7-fold lower levels were observed in the sciatic nerve. **CONCLUSION**: 2-AG is present in the peripheral nervous system, and the levels are markedly higher in cell bodies than those in axons.

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INTRODUCTION

The cloning of the G-protein coupled cannabinoid receptors, CB1 and CB2[1,2], and the demonstration of their abundant distribution in neural and non-neural tissues[3-8] sparked research into the identity and function of this endogenous cannabinoid system. 2-Arachidonylglycerol (2-AG)[9,10] and anandamide[11] were identified as putative endocannabinoids. 2-AG binds to CB1 and CB2 cannabinoid receptors[9,10], inhibits adenylate cyclase activity, produces cannabimimetic effects such as antinociception, hypothermia, and catalepsy[9], and inhibits long-term potentiation in the hippocampus[12]. Hence, 2-AG may be a compound of importance, and the determination of its levels in different biological tissues may offer insights into the functions of the endogenous cannabinoid system. Although its presence in the brain has been determined[9,10,12-14], there have been no reports of its levels in the spinal cord or the peripheral nervous system. Herein, we report a new, improved method for measuring 2-AG using liquid chromatography/ mass

spectrometry (LC/MS), which permitted determination of 2-AG levels in tissues of the peripheral nervous system and the spinal cord.

MATERIALS AND METHODS

Tissue extraction Male Sprague-Dawley rats (330 – 400 g) were anesthetized with urethane (1.25 g/kg ip). In separate animals ($n = 10$), the sciatic nerve at mid-thigh level, the dorsal root ganglion (DRG) at the lumbar spinal level, or the lumbar spinal cord underlying the T13 vertebra was excised. The tissue was dissected free of surrounding connective tissue on ice and weighed. Ice-cold methanol (500 μL for DRG and sciatic nerve, 10 volumes tissue weight for spinal cord) and a small amount of [$^2H_8$]anandamide (Cayman Chemical Company, Ann Arbor, MI) were added to the tissue. The mixture was homogenized on ice for 50 s using a Brinkmann (Westbury NY) Polytron. The homogenate was microcentrifuged for 3 min, and the supernatant was immediately analyzed by atmospheric pressure chemical ionization (APCI) liquid chromatography/mass spectrometry (LC/MS). The time between the start of tissue dissection and application to the LC/MS was (20.8 ± 6.9) min ($\bar{x} \pm s$).

LC/MS analysis of tissue samples Each sample was analyzed for 2-AG using a Hewlett Packard (Palo Alto CA) 1100 series LC/MS. Samples were chromatographed on a pair of 50 mm Zorbax Eclipse XDB- C-18 reversed phase HPLC (4.6 mm id, 1 mL/min) columns with isocratic 85 % methanol, ammonium acetate 1 mmol · L^{-1} and 0.05 % acetic acid. MS conditions, previously optimized using direct flow injection analysis of synthetic 2-AG (RBI, Natick MA), were as follows: selected ion monitoring (SIM) m/z 379.3; fragmentor voltage 70 V; vaporizer temperature 475 ℃; drying gas temperature 200 ℃; drying gas flow rate 7 L/min; corona current 8 μA. Extraction efficiency, estimated by the amount of [$^2H_8$]anandamide recovered in the samples, ranged from 42 % – 92 %. Standard solutions of synthetic 2-AG were analyzed for calibration.

Data analysis Area counts were obtained from peaks at the appropriate retention time according to synthetic standards with the aid of HPChemstation software (Hewlett-Packard). Recovery efficiency was used to correct each sample. Peak areas were converted to 2-AG levels according to linear regression of synthetic standards. Statistical analysis comparing the concentrations (per milligram wet weight) were conducted with analysis of variance (BMDP Statistical Software, Los Angles, CA) followed by comparisons of means using the Newman-Keuls (NK) post hoc test.

RESULTS

With the method developed here, under the same conditions used to analyze the tissue extracts, 2-AG (Fig 1) was detectable at concentrations as low as 20

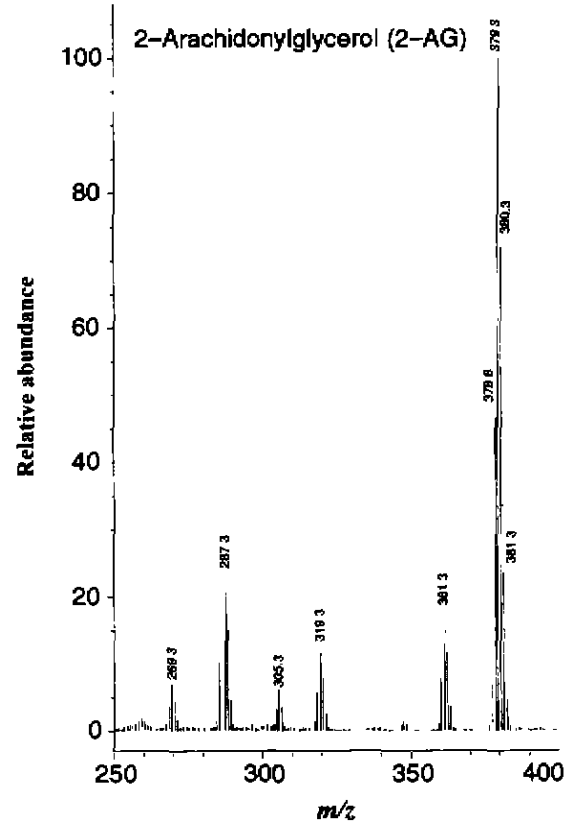

Fig 1. Mass spectrum of 2-AG obtained by APCI-LC/MS. The method, having been developed for maximal sensitivity, produces minimal fragmentation. The major peak centered at m/z 379.3 (the molecular ion of 2-AG, M^+) has the predicted distribution of the naturally-occurring isotopes of 2-AG. The peak at 381.3 is identical to molecular weight of 2-AG M^+ minus water. The second largest peak observed at m/z 287.3 is identical to the molecular weight of 2-AG M^+ minus glycerol.

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fmol with an approximate signal-to-noise ratio of 2 : 1 (Fig 2A). The peak areas exhibited linear increases with increasing concentrations of standards over the concentration range found in the tissue extracts (r = 0.998, Fig 2B).

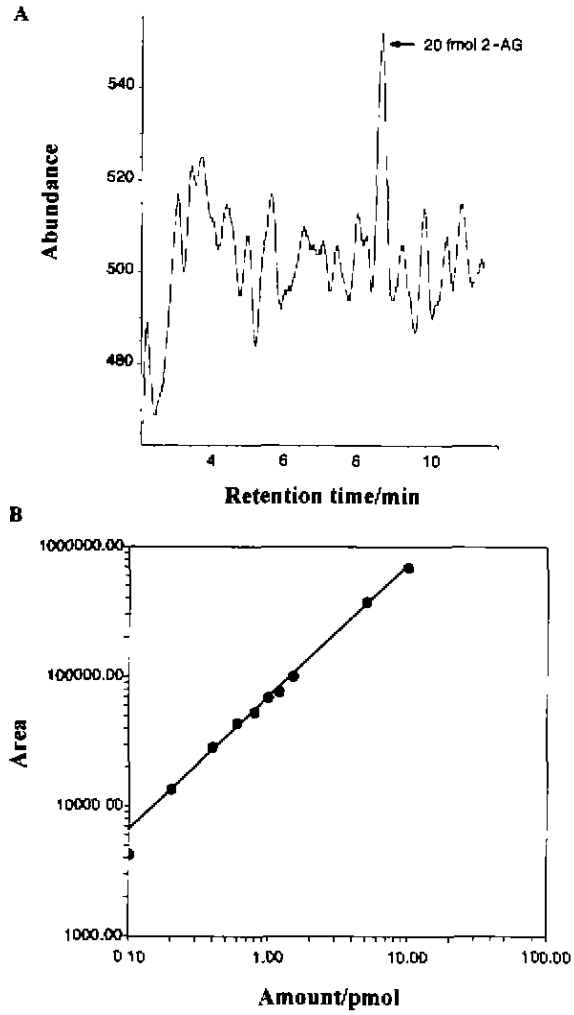

Fig 2. (A) Chromatogram in selected ion monitoring mode at m/z 379.3 showing the elution of 20 fmol synthetic 2-AG with a signal to noise ratio of approximately 2:1. (B) The area counts under 2-AG peaks at the concentration range found in the extract samples showed a high degree of linearity with a correlation coefficient of r = 0.998.

2-AG was detected in all three tissues (Fig 3). As reported previously[9], two chromatographic peaks with similar mass spectra occurred approximately 0.9 min apart. The peak with the shorter retention time corresponded to 2-AG (according to synthetic standards), and the later peak corresponded to the 2-

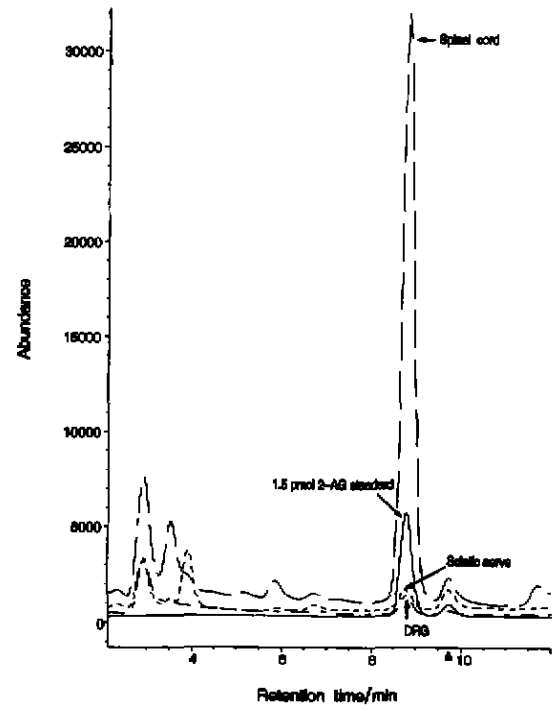

Fig 3. Chromatograms in selected monitoring mode at m/z 379.3 demonstrating the coelution of a 1.5 pmol 2-AG standard with extracts from the lumbar spinal cord, the DRG, and a segment of the sciatic nerve. The identical retention times coupled with the mass selectivity of the instrument provide near-certainty that the compound being detected is endogenous 2-AG. ▲ A peak with similar mass spectra to 2-AG was present, likely corresponding to the 2-AG isomer, 1-AG.

AG isomer, 1-AG. Analysis revealed significant differences in 2-AG levels in the three tissues ($F_{2,7}$ = 6.28, $P < 0.05$, Tab 1). While the concentrations of 2-AG in the lumbar spinal segments and in the DRG did not differ significantly, markedly lower levels were observed in the sciatic nerve compared with either of the other tissues (NK: $P < 0.05$).

Tab 1. Levels of 2-arachidonylglycerol (2-AG) in rat tissues (fmol/mg wet weight). $^bP < 0.05$ (Newman Keuls) *vs* levels in the sciatic nerve.

	n	Mean	SD	SEM
Sciatic nerve	3	52	12.7	7.3
Lumbar spinal cord	4	432^b	185.8	92.9
Lumbar DRG	3	370^b	51.2	29.6

DISCUSSION

The present method for the measurement of 2-AG has several advantages over other existing methods which employed gas chromatography/mass spectrometry (GC/MS). 1) No sample derivatization is necessary, obviating the problem of structural rearrangement of 2-AG when it is derivatized to the bis trimethylsilylether for GC/MS analysis[12]. 2) The LC/MS analysis is rapid, requiring approximately 30 min per sample. 3) The low detection limit of the present method allows reliable quantification of the amounts present in sub-milligram samples of neural tissues.

Previous work suggested the possibility that another endocannabinoid, anandamide, was formed post mortem or by extraction procedures[15,16]. This question has not been addressed systematically for 2-AG. Although the possibility of a similar problem with 2-AG cannot be excluded entirely, it is unlikely that the 2-AG measured here was generated post mortem or *ex-vivo* since the tissues were taken directly from anesthetized animals with minimal time delay from dissection to analysis and with no sample derivatization.

The detection of 2-AG in the lumbar spinal cord, the DRG, and the sciatic nerve is consistent with the presence of cannabinoid receptor markers in these areas. Dense CB1 receptor immunolabeling[17] and CB1 receptor mRNA[18] were found throughout the DRG, and lower levels of immunolabeling were observed in the sciatic nerve, the dorsal root, and the spinal gray[17].

In light of the 7-fold higher concentration of 2-AG in the DRG compared with the peripheral nerve, it appears that the main source of 2-AG is in cell bodies rather than in axons, a possibility not contradicted by the similarly higher levels found in the spinal cord which contains axons, somata, and synapses. The detection of similar levels of 2-AG in the DRG which contains few synapses, and the spinal cord which is rich in synaptic complexity, suggests that terminal boutons are not the major source of 2-AG.

The concurrence of high levels of endocannabinoids, the lack of synapses, and the high lipophilicity of 2-AG (which might permit it to pass through the glial sheaths surrounding DRG cells) suggests the possibility of non-synaptic intercellular communication in the DRG. Alternatively, the endocannabinoid may act on CB1 receptors within the membrane of the cell that synthesizes it. Endocannabinoid binding to CB1 receptors may affect the N-type calcium channels and/or the delayed-rectifying potassium channels that are found on DRG cell bodies[19] and known to be modulated by CB1 receptors[20,21].

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脊髓和周围神经系统中内源性大麻酚类花生四烯甘油的液相层析-质谱检测

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关键词 大麻酚类; 花生四烯甘油; 脊神经节; 坐骨神经; 脊髓; 镇痛; 周围神经系统; 质量光谱分析

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