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Stimulation of central cholinergic neurons by (-)clausenamide *in vitro*[1]

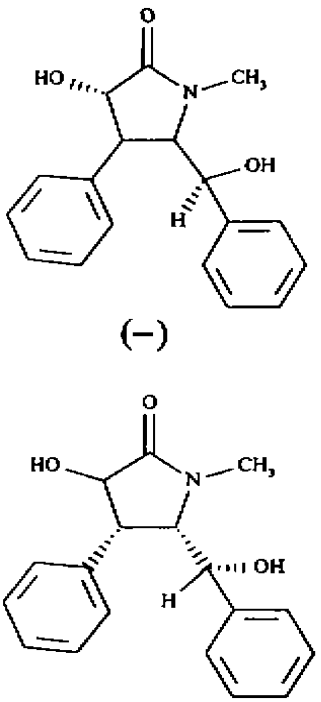
DUAN Wen-Zhen, ZHANG Jun-Tian[2]
(*Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences & Chinese Union Medical University, Beijing 100050, China*)

KEY WORDS clausenamide; cultured cells; choline acetyltransferase; atarabine; frontal lobe; cerebral cortex

AIM: To study the neurotrophic effects of (-) and (+)clausenamide on frontal cortex neurons in culture. **METHODS:** The activity of the choline acetyltransferase (ChAT) was determined by spectrophotometric method; protein content was assayed by Folin phenol method. **RESULTS:** (-)Clausenamide increased the activity of ChAT and protein content in cultured neurons, as well as stimulated proliferation of neuronal cells, support survival and neurite outgrowth of neurons. The neurotrophic action of (-)clausenamide (0.001 - 10 μmol·L^{-1}) was similar to that of nerve growth factor. The (+)clausenamide had no neurotrophic action, even at high concentrations (0.1 - 10 μmol·L^{-1}), but neurons were damaged. **CONCLUSION:** (-)Clausenamide stimulated central cholinergic neuron development.

For a given neuron or neural connection to survive during development, proper contact with the projection area has to be established[1]. such neuron-target interactions are based on the production and release of specific trophic molecules by the target area which are required by the innervating neurons. Some specific trophic molecules, such as nerve growth factor (NGF), brain derived nerve growth factor (BDNGF) support survival or differentiation of central cholinergic neurons[2-3]. Since central cholinergic system accelerates memory process[4], the factors which regulate survival and differentiation of cholinergic neurons may affect ability of learning and memory.

Clausenamide is a compound isolated from *Clausena lansium* (Lour) Skeels[5]. (-) And (+)clausenamide were synthesized in our Institute. Clausenamide, given orally or chronic administration improved learning and memory in step-down and step-through tests. and increased thickness of cerebral cortex and synapses density in the hippocampal CA_3 region[6]. In attempt to clarify the nootropic mechanism of clausenamide, cultured cell of fetal rat frontal cortex was used to observe the effect of clausenamide on ChAT activity and protein content, as well as morphological characteristics of phase-contrast microscopy.



Clausenamide

MATERIALS AND METHODS

Materials (-) And (+) clausenamide (M_r 297) were provided by Department of Medical Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences, purities

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[2] Correspondence to Prof ZHANG Jun-Tian. Phn 86-10-6316-5179. Fax 86-10-6301-7757. E-mail zjtian@public.bta.net.cn
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> 99 %. Acetyl coenzyme A (C 2:0) was Sigma product, its purity is 95 %. Choline chloride was from Sigma Chemical Co, its purity > 99 %. 4, 4'-Dithiodipyridine (4-PDS) was Sigma product, its purity > 99 %. ChAT (EC 2.3.1.6) purified from bovine brain by Sigma Chemical Co was 1.3 kU·g^{-1} solid. NGF was supplied by Chinese Academy of Military Medical Sciences.

Preparation of culture Rat fetuses of embryonic age E15 – 17 were collected into PBS from anesthetized mothers. The brains of the fetuses were excised in DMEM medium without serum. The frontal cortex areas were dissected out. The tissue pieces were washed twice in medium and dissociated by gently pipetting 20 – 30 times through a sterile pipet in 1.5 – 2 mL of medium. 10 mL of medium was added. The supernatant was filtered through a nylon mesh (95 μm). The cells were counted in a hemocytometer using exclusion of trypan blue as criterion for viability. Aliquots of $(6-8)\times10^6$ viable cells were pipetted into wells of 16 mm diameter in 24-well plates (Costar) containing 1 mL of growth medium. Cultured wells were previously coated overnight with a solution of poly-L-lysine (1 g·L^{-1}) in PBS (pH 7.4). The wells were washed 2 – 3 times with sterile PBS before medium was added.

The neurons were grown in DMEM medium with 10 % horse serum and 10 % bovine serum. Cells were incubated at 37 ℃ in 95 % air/5 % CO_2 humidified atmosphere. The medium was changed to DMEM with 5 % horse serum at 24 h after plating and, subsequently, every 2 – 3 d. Each change included 2 rinses of the cells with medium. Drugs were added after 24 h of plating.

Determination of ChAT activity and protein content Cultures were washed with PBS and then homogenized in 250 μL Tris-HCl buffer 50 mmol·L^{-1}, pH 6.0, with 0.3 % Triton X-100, then spun at 300 × *g* for 5 min. The supernatants were taken for determination of ChAT activity[7], and protein content[8]. On d 0, fetal rat frontal cortex neurons were plated in 16-mm well, drugs were added immediately after plating and, cells were taken for ChAT and protein assays at the indicated times.

RESULTS

Morphology After plating for 3 – 4 d, the density of cells in (–)clausenamide and NGF wells were higher than those in control cultures. After 5 d, the cells were confluent. After about 1 wk cultures treated with NGF or/and (–)clausenamide showed very high rates of metabolism requiring daily change of medium to avoid acidification, while (–)clausenamide treated cells were shown densely packed under phase-contrast microscopy, The findings showed that (–)clausenamide supports survival and neurite outgrowth of neurons, stimulate proliferation of neurons. But (+)clausenamide had no neurotrophic effect on cultures, even in higher concentrations (0.1 – 10 μmol·L^{-1}), neurons treated by (+) clausenamide showed necrosis, number of cell decreased (Fig 1).

ChAT activity and protein content In frontal cortex cultures, on d 14, the ChAT activity and protein content were elevated by (–)clausenamide (0.001 – 10 μmol·L^{-1}). (–)clausenamide stimulated proliferation of neuronal cells, while (+)clausenamide (0.1 – 10 μmol·L^{-1}) decreased ChAT activity and protein content (Tab 1).

Tab 1. Effects of (–), (+) clausenamide and NGF on ChAT activity and protein content in cultured frontal cortex cells. Cultures were grown for 14 d. $n = 8$ wells for 6×10^6 cells per well, pooled from 8 rat fetuses of embryonic age E15 – 17. $\bar{x}\pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

NGF, μg·L^{-1}	Clausenamide, μmol·L^{-1}	ChAT activity, pmol ACh·min^{-1}/well	Protein content, μg/well
0		97 ± 16	63 ± 3
2		154 ± 17^c	74 ± 6^c
5		413 ± 18^c	95 ± 18^c
0	(–)10	175 ± 17^c	93 ± 17^c
0	(–)1	163 ± 18^c	89 ± 11^c
0	(–)0.1	138 ± 17^c	81 ± 12^c
0	(–)0.01	126 ± 7^c	75 ± 9^c
0	(–)0.001	117 ± 13^b	69 ± 7^b
0	(+)10	23 ± 1^c	21 ± 3^c
0	(+)1	29 ± 1^b	24 ± 4^b
0	(+)0.1	34 ± 3^c	28 ± 4^c
0	(+)0.01	74 ± 9^c	46 ± 6^c
0	(+)0.001	92 ± 9	61 ± 7
2	(–)0.1	381 ± 21^c	145 ± 12^c
2	(+)0.1	172 ± 16^c	105 ± 11^c

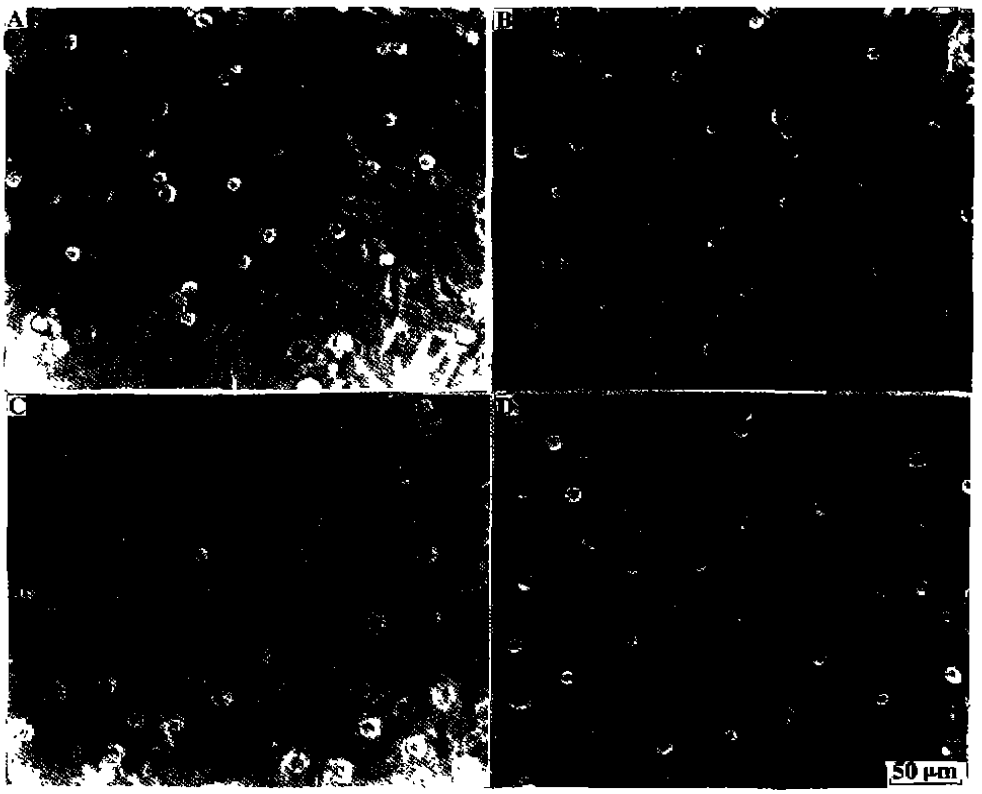

Fig 1. Phase-contrast micrographs of viable frontal cortex cultures grown for 7 d. (A) Without any drug ; (B) with (−)clausenamide; (C) with (+)clausenamide ; (D) with NGF. (×200).

With prolongation of days, ChAT activity and protein content increased gradually in (−)clausenamide and NGF treated wells, while (+)clausenamide treated group showed decline tendency (Fig 2A, 2B).

Neurotropic action of (−)clausenamide and NGF To test whether the effects of drugs on cultured neurons were mediated by glial cells, cell proliferation was inhibited by the addition of atarabine (10 μmol · L^{-1}) which reduced the number of astrocytes without affecting the number of neurons. NGF elevated ChAT activity in cortex neurons in presence or absence of glial cells. Similary, (−)clausenamide increased ChAT activity with or without atarabine, suggesting that the action of neither NGF nor (−)clausenamide depends on the glial cells (Tab 2).

Tab 2. Effects on ChAT in the presence or absence of atarabine 10 μmol·L^{-1}. Cultures were grown.

Group	ChAT activity, pmol ACh·min^{-1}/well	
	No atarabine	Atarabine
Control	79 ± 13	61 ± 8
NGF (5 $\mu g \cdot L^{-1}$)	125 ± 18[c]	118 ± 17[c]
(−)Cla (1 $\mu mol \cdot L^{-1}$)	89 ± 11[b]	69 ± 9[b]

DISCUSSION

It is well established that the growth and differentiation of forebrain cholinergic neurons is stimulated by NGF[9-11]. The present study suggests that (−)clausenamide but not (+)clausenamide exert similar actions. The findings showed that there are significant differences in actions of (−) and (+)clausenamide.

(−)Clausenamide stimulated the synthesis of protein and ChAT. Treatment of the cultures with ara-C could prevent cells proliferation only slightly diminished the neurotrophic effects of (−)clausenamide. These effects suggested that (−)clausenamide elevates ChAT activity in cultures by a direct action on neurons and, to a

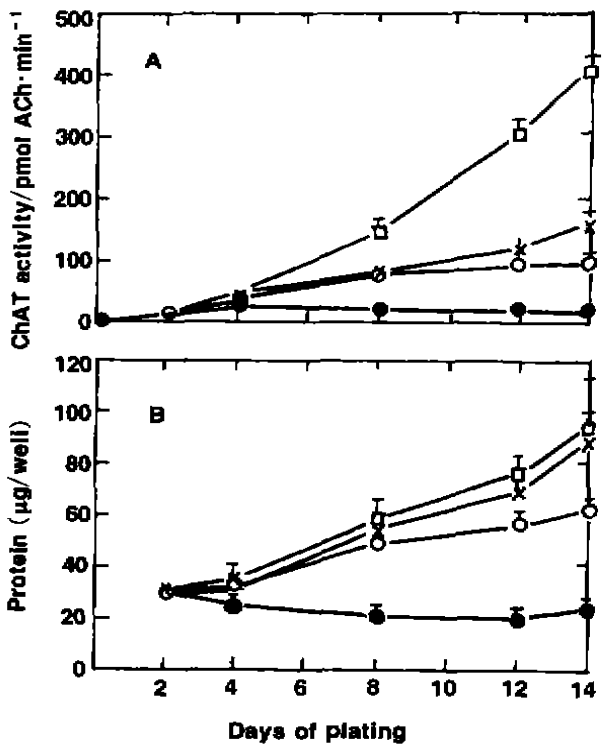

Fig 2. Effects on ChAT activity (A) and protein content (B) in cultured cortex neurons. $n = 8$ wells, $\bar{x} \pm s$. ○ Control; ● (+) clausenamide; × (−) clausenamide; □ NGF.

minor extent, by indirect action via stimulation of glial cell proliferation.

Any indirect stimulations mediated by glial cells or non-cholinergic neuronal cells was not involving NGF in cultures[12], therefore, the neurotrophic actions of NGF and (−) clausenamide on frontal cortex neurons were mediated by different mechanism.

Central cholinergic neurons have a higher intrinsic plasticity to different trophic or specifying factors and certain treatments[13]. Obviously, such neural plasticity is particularly interesting, since central cholinergic mechanisms have been recognized to be instrumental in memory process[14]. Our findings that (−) clausenamide increase ChAT activity of neurons and stimulate development of frontal cortex cultures will finally lead to increment of synaptic structural plasticity and facilitation of learning and memory, this is benefit to elucidate its nootropic mechanism.

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(−)黄皮酰胺体外对胆碱能神经元生长的促进作用

R962

段文贞，张均田[1]
(中国医学科学院药物研究所药理室，北京 100050，中国)

关键词 黄皮酰胺；培养的细胞；胆碱乙酰转移酶；阿糖胞苷；前脑叶；大脑皮质

目的：研究左旋，右旋黄皮酰胺对培养脑皮层神经元发育有无促进作用. 方法：用比色法测定胆碱乙酰转移酶(ChAT)活性，用 Folin 酚法测定蛋白含量，细胞生长发育状态在倒置相差显微镜下动态观察. 结果：左旋黄皮酰胺(0.001 – 10 μmol·L^{-1})能促进皮层神经元细胞发育，光镜下看到细胞密度增加，突触生长旺盛；培养细胞中 ChAT 活性及蛋白含量较对照组明显增高. 右旋黄皮酰胺却无神经营养作用，且在高浓度时对培养神经元有损伤作用. 结论：左旋黄皮酰胺促进中枢胆碱能神经元发育，易化突触结构的可塑性.

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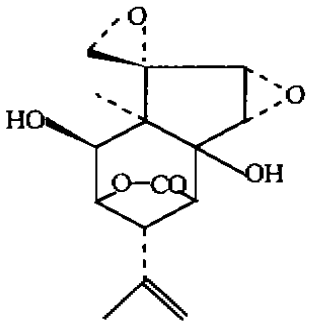
Effect of *Coriaria* lactone on cytosolic free calcium of cultured neurons from rat cerebral cortex[1]

ZHU Xiao-Feng[2], ZHAO Xi-Long[3], ZHU Chang-Geng (*Department of Brain Research, Tongji Medical University, Wuhan 430030, China*); WANG Jin-Xi (*State Key Laboratory of Coordination Chemistry, Nanjing University, Nanjing 210093, China*)

KEY WORDS *Coriaria*; lactones; neurons; cultured cells; calcium; cerebral cortex

AIM: To study the effect of *Coriaria* lactone (CL) on cytosolic free calcium ($[Ca^{2+}]_i$) of cultured neurons from cerebral cortex. **METHODS**: Primary neuron culture (14 d) and AR-CM-MIC cation measurement system were used, the $[Ca^{2+}]_i$ were measured. CL effect was observed by loading egtazic acid. **RESULTS**: The $[Ca^{2+}]_i$ of cultured neurons (99.4 – 103.4) nmol · L^{-1} was elevated concentration-dependently by CL (25 – 500) μmol·L^{-1} ($P < 0.01$). This effect disappeared after loading egtazic acid 5 mmol · L^{-1}, but reappeared after adding $CaCl_2$ to 1 mmol · L^{-1}. **CONCLUSION**: The $[Ca^{2+}]_i$ of cultured neurons was elevated by CL, depending on extracellular Ca^{2+}.

Epilepsy is a common disorder. The animal models evoked by *Coriaria* lactone (CL) was suitable to study the epileptogenesis[1]. The elevation of cytosolic free calcium ($[Ca^{2+}]_i$) is the key step in the process of neuronal injure and death[2]. Calcium regulation is a core problem in the study of epileptogenesis and the results were analysed easily using the cultured neurons. The present study was designed to investigate the effect of CL on $[Ca^{2+}]_i$.



Coriaria lactone

MATERIALS AND METHODS

Agents and equipments CL (West China Medical University Pharmaceutical Factory, Lot № 8338, purity 98 %, pH 3.5 – 5.5, melting point 211 – 2 ℃, $[\mu]_D$ + 10, $C_{15}H_{18}O_6$). AP_5, verapamil, and Fura 2-AM (Sigma Co). AR-CM-MIC cation measurement system (Spex Co). Diapho-TMD fluorescence microscope

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[2] Correspondence to Prof ZHU Xiao-Feng. Now in *the Second Affiliated Hospital, Medical College, Jiamusi University, Heilongjiang 154002, China*. Phn 86-454-822-8003. Fax 86-454-867-1733.

[3] Now in *Institute of Environment and Health Monitoring, Chinese Academy of Preventive Medicine, Beijing 100021, China*.

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