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Stimulatory effect of lipophilic extracts of *Epimedium macranthum* on PC12h cells

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Abstract

Stimulatory effect of the diethyl ether and ethyl acetate extracts of Inyokaku (淫羊藿), *Epimedium macranthum* MORR. et DECNE. (大連; China, yín yáng huò) on cultured pheochromocytoma cells, PC12h cells, inducing their differentiation to neuronal cells, was studied. Cultivation of cells with the extracts (0.01-0.1 mg/ml of medium) for 3-5 days promoted the outgrowth of neurites from the cells in a dose-dependent manner and enhanced the activity of their voltage-dependent Ca^{2+} channel and response to carbachol as detected by elevation of the cytoplasmic free calcium level. Pre-treatment of cells with the acetate extract for several days increased greatly the muscarinic binding density.

Key words *Epimedium macranthum*, lipophilic extract, PC12 cell, neurite outgrowth, Ca^{2+} channel, muscarine receptor.

Abbreviations E.m., *Epimedium macranthum* MORR. et DECNE; DMEM, Dulbecco's modified Eagle medium; NGF, nerve growth factor; QNB, quinuclidinyl benzilate.

Introduction

Inyokaku (yín yáng hòu), the herb of *Epimedium* species, has been used as a component of Kampo prescriptions such as Inyokaku-tou (淫羊藿湯), Yokaku-sansi-to (羊藿三子湯) and Saniku-tan (贊育丹), and is believed to be effective in therapy of impotence, amnesia, asthenia, or correspondent symptoms.[1] In a few experimental reports on the pharmacology of the crude extracts of the herb, inhibition of the angiotensin-converting enzyme,[2] stimulation of phagocytotic action,[3] and positive inotropic effect on the isolated guinea pig heart[4] have been demonstrated.

Since we are so interested in the active ingredient (s) of Inyokaku responsible for its anti-amnestic effect, elucidation of neurotrophic actions has been attempted for its lipid-soluble components, which are assumed easily to penetrate the plasma membrane of the cells, and also the intestinal mucosa as well as the blood-brain barrier, to be absorbed into the action sites.

PC12 cell is a para-neuronal culture cell having its origin in the rat adrenal pheochromocytoma and useful as an undifferentiated cell model senditive to nerve growth factor (NGF)[5,6] and other neurotrophic agents[7-9] to differentiate to nerve cell, extending neurites, expressing receptors for neurotransmitters such as acetylcholine and activating the Ca^{2+} channels.

Stimulatory effect of the lipophilic extracts of Inyokaku is reported in this communication on a subline of PC12 cell with special reference to their neurite-and differentiation-inducing actions as determined by morphological change and increase of carbachol sensitivity and potential-dependent calcium influx.

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Materials and Methods

Chemicals : [^{3}H] Quinuclidinyl benzilate (1.85 TBq/mmol) was purchased from Amersham. Fluo-3 acetoxymethyl ester was purchased from Dojin Chemicals Co., Japan.

Preparation of herb extracts : *Epimedium macranthum* MORR. et DECNE. (E.m.) was purchased from Tochimoto Tenkaido Co., Ltd., Japan. One hundred g of the aerial parts of E.m. was extracted with a total of 4.8 l (1.6 l×3) of water by successive boiling. The combined filtrate was concentrated to 1/5 of the volume, and the resulting substance was extracted with 700 ml diethyl ether at room temperature. The ether layer was evaporated to dryness to give 81.9 mg of paste extract. The water residue was extracted with 700 ml of ethyl acetate to give 1.08 g of the ester extract. Each extract was dissolved in dimethyl sulfoxide to obtain 500-1000 times concentrated stock solutions for tests.

Cultivation of PC12h cells : PC12h cells, a subclone of PC12 cell established by Hatanaka,[10] were cultured as described previously.[11] The medium used was Dulbecco's modified MEM (DMEM) supplemented with 5 % (v / v) horse serum and 5 % (v/v) precolostrum newborn calf serum. Two to 18 test plates, 35 mm collagen-coated plastic plates, were applied to each group of treatment as indicated in the results.

Measurement of neurite outgrowth : Total area of the neurites per cell was figured as an average of 25-30 cells in about 10 areas of two culture plates for each group in successive days of treatment with the test extract or dimetyl sulfoxide (control) using a computed image processor (XL-500, Olympus, Japan) attached to a phase-contrast microscope. The stock solutions of extract were added to the culture medium in various final concentrations as specified in the results at day 1 of treatment.

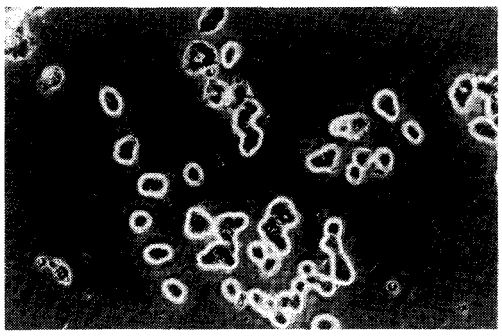
Measurement of cytoplasmic free calcium concentration : Free calcium concentration of individual cells was monitored by loading them with a specific fluorescent dye, Fluo-3, before assay as described previously.[11] Cells cultured in 35 mm culture plates were pretreated with the diethyl ether or ethyl acetate extract (0.1 mg/ml) for 5 days as specified in the results. They were incubated with Fluo - 3 acetoxymethyl ester in HEPES - buffered Krebs medium (in mM, 124 NaCl, 5 KCl, 1.24 KH_2PO_4, 1.3 $MgSO_4$, 2.46 $CaCl_2$, 10 glucose, 10 HEPES/TRIS ; pH 7.4) for 90 min. Following incubation for free dye formation and washing, they were exposed to carbachol (0.1 mM) or 40 mM KCl by perfusion of the test solution dissolved in HEPES - buffered Krebs medium into the culture plates maintained at 30°C for about 10 min. The change in fluorescence intensity (emission at 530 nm with excitation at 490 nm) of individual cells before and immediately after addition of the stimulators was recorded at intervals of 20 sec by an argon laser-equipped fluorocytometer, ACAS 570 (Meridian Instruments Co., U.S.A.).

Determination of muscarinic ligand binding sites : Cell cultures in 35 mm plates were subjected to determination of binding of quinuclidinyl benzilate (QNB), a potent muscarinic antagonist. Cells of each plate were treated with the ethyl acetate extract (0.05 mg/ml of culture medium) or the vehicle (control) for 3-4 days after inoculation of cells (2×10^5 cells per plate) and 24 h of culture. They were washed with HEPES-buffered DMEM (bicarbonate-free) (pH 7.4) before binding assay, and then incubated with the medium containing 1.0 nM [^{3}H] QNB (1.85 KBq/ml) for 10 min at room temperature (18-23°C). About half of the plates of each control and extract group were incubated with QNB in the presence of 1.0 μM atropine to estimate the atropine sensitive fraction of total binding that was obtained from plates incubated without the inhibitor. Cell layers were washed twice with cold phosphate-buffered saline after incubation and harvested with 1.0 ml/plate of 0.5N NaOH solution. Each 0.5 ml of the harvest was subjected to radioactivity counting with a conventional toluene-Triton scintillater in a vial. Protein of each sample was estimated by determination of it for an aliquot of the harvest according to the method of Lowry *et al.*[12] using bovine serum albumin standard.

Results

Fig. 1 shows morphology of the culture of PC12h cells at 6 days of control (a) and diethylether extract (0.028 mg / ml) treatment (b). Cells became polygonal and flattened the next day of culture following addition of the extract, and extended progressively neuritic processes with days of treatment to make mutual networks.

a



b

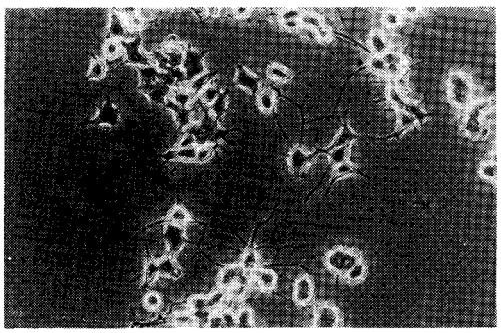


Fig. 1 Morphological changes of PC12h cells due to treatment with the diethyl ether extract of *Epimedium macranthum*. Cells cultured in 35 mm culture plates were treated with 0.028 mg/ml of the extract (b) or vehicle (a) for 6 days. (Phase-contrast, ×300)

Growth of the neuritic processes following addition of the ether extract (0.01-0.1 mg/ml) is expressed as the average area of neurites per cell on the image display of the phase-contrast microscopy up to 5 days of culture in Fig. 2,

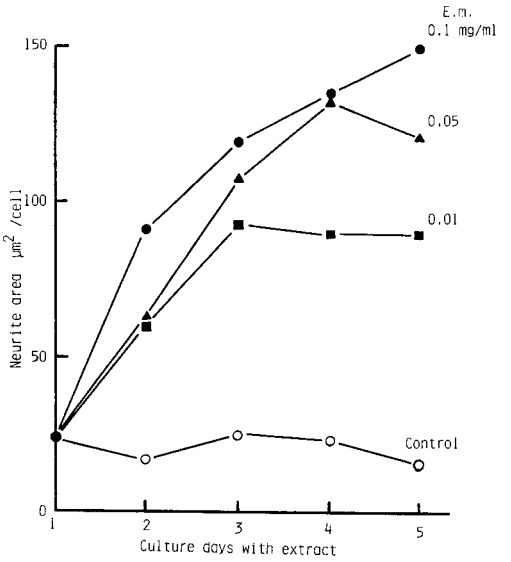


Fig. 2 Outgrowth of neurites after addition of the diethyl ether extract to PC12h cells.
Cells were inoculated at $1\text{-}2\times10^4$ in 2 ml medium in each 35 mm culture plate and cutured with addition of the indicated concentrations of the extract or vehicle (control) to medium at day 1. Method of evaluation of the outgrowth of neurites (increase of neurite area per cell) is as described in Materials and Methods. Each point is the mean of 25-30 events.

showing a dose-dependent increase of the average area in the terminal phase of growth. Similarly the ethyl acetate extract was shown to promote the neurite outgrowth in a concentration range from 0.01 to 0.1 mg/ml (data not shown).

Responses of individual cells in the cytoplasmic free calcium concentration to the stimulation with 0.1 mM carbachol and 40 mM KCl are shown in Figs. 3 and 4, respectively, as measured by increase of the fluorescence intensity of the Ca^{2+}-specific fluorescent dye entrapped in the cells after 5 days of treatment with 0.1 mg/ml of the ether extract. Parts of the cells were found insensitive to carbachol and high KCl in both control and extract-treated groups. Responses of cells to the stimulators after 5 days of treatment with 0.1 mg/ml of the ethyl acetate extract were also recorded (data not shown).

Statistic evaluation of the differences of the responses to carbachol and KCl between control

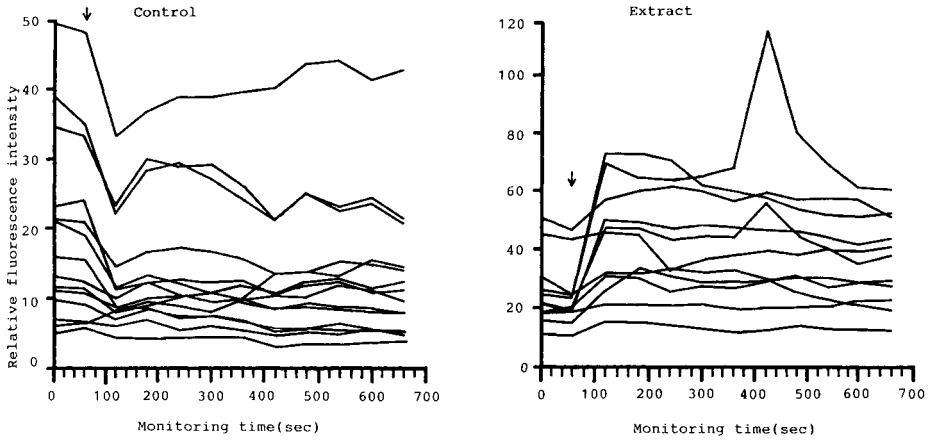


Fig. 3 Effect of the diethyl ether extract on cytoplasmic free Ca^{2+} elevation induced by stimulation with carbachol. Cells were pretreated with the ether extract (0.1 mg/ml) or vehicle (control) in culture medium for 5 days. Cells loaded with Fluo-3 were superfused with HEPES-buffered Krebs medium (pH 7.4) containing 0.1 mM carbachol for more than 10 min. Fluorescence intensity at 530 nm of individual cells (12 or more per group) before and after exposure at arrows to the stimulator was chased with a fluorocytometer.

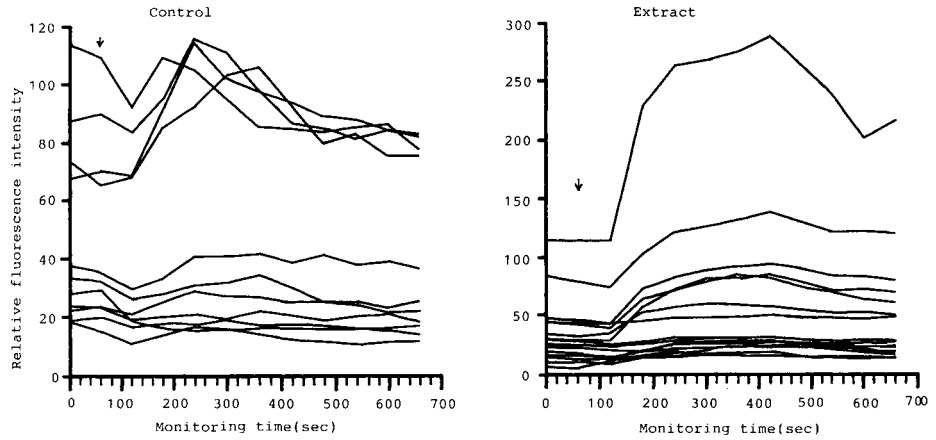


Fig. 4 Effect of the diethyl ether extract on cytoplasmic free Ca^{2+} elevation induced by stimulation with 40 mM KCl. The method is essentially the same as described in the legend to Fig. 3, except for 40 mM KCl instead of carbachol.

and the extract-treated groups is shown in Table I. As determined by the averaged ratio of the maximal fluorescence intensity after addition of one of the stimulators to the intensity before addition in each group, the free calcium level in PC12h cells was elevated by stimulation with carbachol and high KCl (membrane depolarization) much more in groups treated with either of the diethyl ether and ethyl acetate extracts than in control groups as an indication of functional differentiation of the cells by the treatment. Calcium accumulation on addition of carbachol was

Table I Statistic evaluation of the effect of the extracts on cytoplasmic free Ca^{2+} elevation induced by stimulation with carbachol or 40 mM KCl.

Extract	Stimulator	Fluorescence enhancement after stimulation mean±SEM			P*
		Control	Treatment	Increase %	
diethyl ether	carbachol	1.29±0.05 (16)	2.08±0.28 (13)	61.2	<0.01
	KCl	1.38±0.05 (12)	2.01±0.20 (20)	45.7	<0.01
ethyl acetate	carbachol	1.15±0.07 (19)	2.01±0.26 (9)	74.8	<0.01
	KCl	1.43±0.06 (12)	1.94±0.13 (11)	35.7	<0.01

PC12h cells were treated with either the dietyl ether or ethyl acetate extract (0.1 mg/ml) for 5 days before stimulation as indicated in the table. These results are based on the data shown in Figs. 3 and 4, except for the ethyl acetate extract. Fluorescence enhancement due to stimulation of cells was expressed as the ratio of the maximal intensity after addition of the stimulator to intensity just before addition of it for each cell.
*Probability calculated by the non-parametric test according to Wilcoxon for means of control and treatment groups. Figures in parentheses are numbers of cells measured.

Table II Effect of the ethyl acetate extract on atropine-sensitive QNB binding to PC12h cells.

Exptl. No.	Treatment		n	Binding mean±SEM fmol QNB/mg protein	p
	Days	Group			
1	3	C	4	127±53	n.s.*
		E	10	223±37	
2	3	C	6	103±15	<0.01**
		E	6	266±80	
3	3	C	6	45±16	n.s.*
		E	8	69±15	
4	3	C	3	70±13	n.s.*
		E	3	133±39	
5	4	C	9	54±10	<0.05*
		E	10	132±31	

Cells were treated with (E) or without (C) extract (0.05 mg/ml) for 3 or 4 days before binding assay. Total binding of QNB to cells was determined by incubation of numbers of culture plates (n) indicated for each group with [^{3}H] QNB in HEPES-buffered DMEM for 10 min, and atropine-insensitive binding by incubation of corresponding numbers of plates in the presence of 1.0 μM atropine sulfate in medium. Difference between the total and atropine-insensitive (average) bindings was assumed to be the sensitive binding.
*Significance between means of control (C) and extract-treated (E) groups by Student's *t*-test. n.s. : Not significant.
**Significant by the non-parametric test according to the method of Wilcoxon between control and treatment groups.

almost all atropine-susceptible in individual cells of both control and extract-treated groups (data not shown).

Atropine-sensitive QNB binding (10-40 % of total binding) was compared between control and the ethyl acetate-treated cells in repeated experiments (Table II). Since the total binding of QNB obtained by incubation of dishes of cells, control and extract-treated, with the radio-labeled substrate is thought to include non-specific absorption by the cells, the receptor - bound fraction (muscarine receptor binding) of it was calculated by subtracting the residual binding determined in the presence of 1.0 μM atropine from the total for the control and treated cells. Differences of the averages of binding between the control and extract-treated groups were significantly large in proof of the stimulatory effect of the ester extract in some of the experiments as shown in the table. The averages of QNB binding were generally larger in the extract-treated groups than in the controls also in the other experiments.

Discussion

The diethyl ether and ethyl acetate extracts of *Epimedium macranthum* MORR. et DECNE. notably activated PC12h cells to promote the neurite outgrowth and amplify the action of acetylcholine analogue on the receptor-mediated elevation of free Ca^{2+} in the cells and the depolarization-induced Ca^{2+} uptake. Differentiation of cells treated with the extracts for 3 days or more has been confirmed by increase in atropine-sensitive QNB binding density in whole cells, although total binding of QNB was found to be indifferent between control and extract - treated samples (data not shown).

A marked increase of atropine - sensitive QNB binding or muscarinic receptors has been found in PC12 cells[13] after treatment with NGF, as well as promotion of the outgrowth of neurites,[14] differentiation and survival in the brain and peripheral neurons.[15-18] It has also been reported that NGF can dramatically affect the pharmacological type of the high K^+-sensitive Ca^{2+} channels of PC12h cells.[19] Therefore one or several of components of the lipophilic extracts of *Epimedium macranthum* are expected to possess the potential to stimulate neurons and promote the extension of neural network in the nervous tissues. Extension of the neuronal process is fundamental to establishment of the intricate network of the nervous system and fulfilment of its specific functions. In fact induction of tyrosine hydroxylase[10] and choline acetyltransferase,[20, 21] and differentiation of several ion channels[19] have been found after the addition of NGF to NGF-sensitive neuronal cells. Other growth factors such as cAMP, epidermal growth factor and fibroblast growth factor have also been shown to stimulate pheochromocytoma or neuroblastoma cells to elicit neurite outgrowth,[7, 22-24] to increase ornithine decarboxylase activity[25] or to induce early specific mRNA.[9] The neurotrophic activity of the above mentioned various growth factors seems to be linked to their capability of maintaining neuronal function as well as supporting resistance to change in environmental conditions, e.g. glutamate neurotoxicity,[26, 27] ischemia,[28] lipid peroxidation (or generation of O_2 radicals),[29] brain injury,[17] *etc.*

So far the mechanism of the neurotrophic effect of the herb lipophilic extracts is unknown. Although the neurite - promoting effect of the extracts could be observed as early as within 24 h after the addition of them to the medium in consistence with that of NGF, the effect attained almost the maximum by 4 or 5 days of treatment (cf. Fig. 2), irrespective of renewal of the medium, and the processes (two or more) were not so different in length in each cell, axon-like prominent one seldom appearing. Therefore not only the possibility of enhancement of NGF production by PC12h cells as a result of treatment with the herb extracts, but also common steps with NGF or other growth factors in the action mechanism should be investigated in further experiments. It would also be very interesting to check whether the lipophilic component (s) of the herb has activities to maintain the neurons and enhance the psychoneuropharmacological responses of aged animals.

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和文抄録

淫羊藿のジエチルエーテル，あるいは酢酸エチル・エキスはラット副腎褐色細胞腫由来のPC12h細胞を刺激して，これを神経細胞に分化させることが示された。この細胞をこれらエキス（0.01～0.1 mg/ml）と3～5日間培養すると，用量依存的に神経突起の成長が促進され，また高濃度KClによる脱分極刺激，あるいはカルバコールに反応して，細胞内遊離カルシウムレベルの上昇が観察された。細胞を数日間酢酸エチル・エキスで処理すると，ムスカリン・タイプの基質結合が著増することが分かった。

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