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Effects of Choline and Inositol on ^{3}H-Dihydroalprenolol and ^{125}I-Iodocyanopindolol Bindings to β-Adrenergic Receptors of the Guinea Pig Cerebral Cortical Membranes

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This experiment was designed to examine the effects of choline and inositol in Eagle's minimal essential medium (MEM), because the values of specific binding were increased when the ^{3}H-dihydroalprenolol (^{3}H-DHA) binding assay of β-adrenoceptors in the guinea pig and rat cerebral cortical membranes was carried out in Eagle's MEM instead of 60 mM Tris–HCl, 20 mM $MgCl_2$ buffer (pH 7.4). All chemicals contained in the MEM were added to the ^{3}H-DHA binding mixture and the values of the specific binding were compared with the control values obtained in the absence of the chemicals. The presence of choline (0.1—10 μM) or inositol (0.1—10 μM) induced an increase of the values of the specific binding. These effects of choline and inositol were observed after pre-incubation for 20 and 1 min, respectively. After 30 min of incubation, no changes were observed upon removal of these chemicals. Furthermore, choline and inositol significantly increased the β-adrenoceptor density (B_{max}) when ^{3}H-DHA and ^{125}I-iodocyanopindolol were used as the radioligands, as determined by Scatchard analysis, but no significant change was observed in the value of the dissociation constant (K_d) in ^{3}H-DHA binding. These results suggest that choline and inositol in the membranes could have a crucial role in drug–receptor interaction.

Keywords——β-adrenoceptor; inositol; choline; binding assay; Eagle's minimal essential medium

It is well known that choline and inositol are major components of phospholipid in the membranes. On the other hand, choline is a precurser of phosphatidylcholine, which influences the membrane fluidity and enzyme activity in the membranes. Inositol is also a main metabolic product from phosphoinositide turnover. These chemicals are contained in Eagle's minimal essential medium (MEM), which is now widely used for cell or tissue culture or cell proliferation.

We reported[1] that changes in the negative or positive charges of the cell surface by the addition of polymeric effectors such as polymyxin B, heparin or deoxyribonucleic acid (DNA) induced alterations in the affinity of ^{3}H-dihydroalprenolol (^{3}H-DHA) to β-adrenoceptors in rat brain, suggesting that the charges in the carbohydrate chains, phospholipids and protein participate in the drug–receptor interaction. We also reported[2] that the carbohydrate chains in cell membranes are implicated in the drug–receptor interaction of β-adrenoceptors based on the results obtained by the addition of lectins and the treatment of the membranes with glycosidases and glycopeptidase.

Thus, as most investigators who intend to examine functional receptor mechanisms use Tris–HCl buffer (pH 7.4) for radioligand binding assay, the present study was carried out to test the effects of choline and inositol contained in Eagle's MEM on ^{3}H-DHA or ^{125}I-iodocyanopindolol (^{125}I-ICYP) bindings to the β-adrenoceptors in the cerebral cortex of guinea pig.

Experimental

(−)-^{3}H-DHA (104.8 Ci/mmol) and (−)-^{125}I-ICYP (2200 Ci/mmol) were purchased from New England Nuclear Corp. Eagle's MEM, choline and inositol were used.

The membrane-enriched fraction from the guinea pig and rat brain was prepared by using the following methods. Male Wistar rats weighing 300—400 g and male Hartley guinea pigs weighing 350—500 g were used in this study. After removal of the guinea pig brain and rat brain, cerebral cortex from each (about 1—1.5 g) was minced and homogenized in 10 volumes of 10 mM Tris–HCl, 0.25 M sucrose, pH 7.6, with a glass homogenizer. The homogenates were filtered through 4 layers of gauze, and the filtrates were centrifuged at 40000 *g* for 30 min. The resultant pellets were rinsed at once with 120 mM Tris–HCl, 40 mM $MgCl_2$, pH 7.4 or with Eagle's MEM (pH 7.4) and homogenized in 20 ml of the same buffer with a glass homogenizer. The membrane-enriched fraction was frozen in liquid nitrogen, stored at −80°C, and, immediately before use, further diluted to the appropriate concentrations as indicated below in the text. During 2 months' storage there was no apparent decrease in antagonist binding. Protein concentrations were determined by the method of Lowry *et al.*[3] using bovine serum albumin as the standard.

The β-adrenoceptor binding assay was carried out in duplicate with ^{3}H-DHA or ^{125}I-ICYP. For ^{3}H-DHA binding, 0.25 ml of membrane suspension (0.25 mg of protein) was incubated for 30 min at 23 °C with 1.2 nM ^{3}H-DHA and various concentrations of effectors such as inositol in a total volume of 0.5 ml containing 60 mM Tris–HCl and 20 mM $MgCl_2$ (pH 7.4). For ^{125}I-ICYP binding, 0.25 ml of membrane suspension (0.1 mg of protein) was incubated for 45 min at 23 °C in a total volume of 0.5 ml containing the same buffer. In some cases, Eagle's MEM was used. In the ^{3}H-DHA binding and ^{125}I-ICYP binding saturation experiments, the concentrations of ^{3}H-DHA and ^{125}I-ICYP were 0.1—10 nM and 0.01—1.0nM, respectively. At the end of the incubation period, the incubation medium was immediately filtered through a Whatman GF/C glass fiber filter using the improved method.[4] In the case of ^{3}H-DHA binding, the filter was added to 5 ml of Tt 76 scintillation fluid and the radioactivity was counted with the scintillation counter. In the case of ^{125}I-ICYP binding, the radioactivity of the filter in a tube was counted with an auto-well gamma counter. The difference in mean values between total and non-specific binding determined in the presence of 10 μM *l*-propranolol was taken as the specific binding. The specific bindings with ^{3}H-DHA and ^{125}I-ICYP routinely amounted to approximately 80% and 85% of the total binding, respectively.

All kinetic analyses were carried out on an NEC PC-9801F computer system that performed iterative non-linear regression as described previously.[5]

In order to quantitate the mode of saturation, Hill numbers of ^{3}H-DHA and ^{125}I-ICYP bindings to the membranes were determined by the use of the Hill plot.[6]

The significance of differences was analyzed by using Student's *t*-test or the paired Student's *t*-test.

Results

When the ^{3}H-DHA binding assays of β-adrenoceptors in the guinea pig and rat cerebral cortical membranes were carried out in Eagle's MEM (pH 7.4) instead of 60 mM Tris–HCl, 20 mM $MgCl_2$ buffer (pH 7.4), the values of specific binding were increased: 34.69±7.56% ($n=3$, $p<0.05$) in the rat brain and 22.36±7.59% ($n=6$, $p<0.05$) in the guinea pig brain (Table I).

Table II summarizes the composition of Eagle's MEM and the effects of the components on ^{3}H-DHA binding to β-adrenoceptors from guinea pig. Choline and inositol significantly increased the specific binding, while L-histidine and L-tryptophan significantly reduced the specific binding. Other chemicals had no effect on the ^{3}H-DHA binding. The effects of choline

TABLE I. Effects of Eagle's MEM on ^{3}H-DHA Binding to Cerebral Cortical Membranes from Guinea Pig and Rat

	Specific binding (fmol/mg protein)		% of control
	Control	MEM medium	
Rat brain (3)	51.87±2.91	69.47±3.44[a]	134.69±7.56
Guinea pig brain (6)	15.87±0.85	19.15±0.55[b]	122.36±7.59

The control binding assay was carried out in the buffer of 60 mM Tris–HCl, 20 mM $MgCl_2$ (pH 7.4) using 1.2 nM ^{3}H-DHA as a radioligand. Values are means±S.E. Numbers in parentheses represent the numbers of experiments. Student's *t*-test, *a*) $p<0.001$ *vs.* control, *b*) $p<0.02$ *vs.* control.

TABLE II. Composition of Eagle's MEM and Effects of the Components on β-Adrenoceptor from Guinea Pig Brain

	mg	mM	% of control
NaCl	6800	116.36	
KCl	400	5.36	
NaH_2PO_3	115	0.96	
$MgSO_4$	93.5	0.78	100.99± 7.31 (3)
$CaCl_2$	200	1.80	
Succinic acid	75	0.41	
Sodium succinate	100	0.40	
Glucose	1000	5.56	109.43± 9.82 (3)
L-Arginine·HCl	126	0.60	100.17± 4.28 (3)
L-Cysteine·HCl·H_2O	31.4	0.18	101.39±20.21 (3)
L-Tyrosine	36	0.20	98.66± 4.05 (3)
L-Histidine·H_2O	42	0.22	81.64± 6.28 (3)[a]
L-Isoleucine	52	0.40	103.08±14.41 (3)
L-Leucine	52	0.40	97.53± 8.51 (3)
L-Lysine·HCl	73	0.40	100.50± 1.73 (3)
L-Methionine	15	0.09	99.81±17.39 (3)
L-Phenylalanine	32	0.19	100.75± 4.92 (3)
L-Threonine	48	0.40	101.17± 3.04 (3)
L-Tryptophan	10	0.05	91.19± 4.14 (3)[a]
L-Valine	46	0.39	98.67± 6.93 (3)
Choline bitartrate	1.8	0.007	126.81± 9.54 (5)[a]
Folic acid	1	0.002	106.05±13.75 (3)
Inositol	2	0.01	116.84± 2.76 (4)[b]
Nicotinamide	1	0.008	102.91± 9.59 (3)
Calcium pantothenate	1	0.002	104.82± 2.36 (3)
Pyridoxal·HCl	1	0.005	92.97±15.86 (3)
Riboflavin	0.1	0.0003	96.71± 5.70 (3)
Thiamin·HCl	1	0.003	104.08±11.82 (4)
Biotin	0.02	0.00008	100.70±11.81 (3)
Kanamycin	60	0.12	ND
Phenol red	6	0.017	ND

The control binding assay was carried out in the buffer of 60 mM Tris–HCl, 20 mM $MgCl_2$ (pH 7.4) using 1.2 nM ^{3}H-DHA as a radioligand. Values are means±S.E. Numbers in parentheses represent the numbers of experiments. Student's *t*-test, *a*) $p<0.05$ *vs.* control, *b*) $p<0.001$ *vs.* control. ND means not determined.

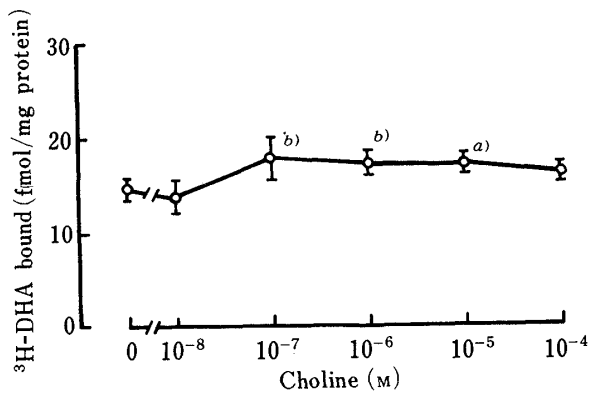

Fig. 1. Effects of Choline on β-Adrenoceptor Binding

The data represent the mean values±S.E. of duplicate determinations from five separate experiments. Paired Student's *t*-test: *a*) $p<0.05$ or *b*) $p<0.02$ *vs.* without choline.

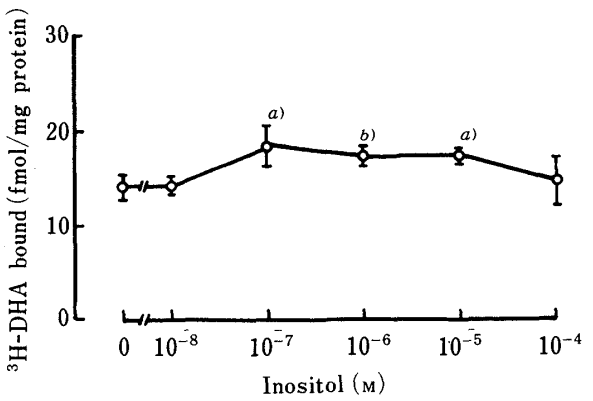

Fig. 2. Effects of Inositol on β-Adrenoceptor Binding

The data represent the mean values±S.E. of duplicate determinations from five separate experiments. Paired Student's *t*-test: *a*) $p<0.05$ or *b*) $p<0.01$ *vs.* without inositol.

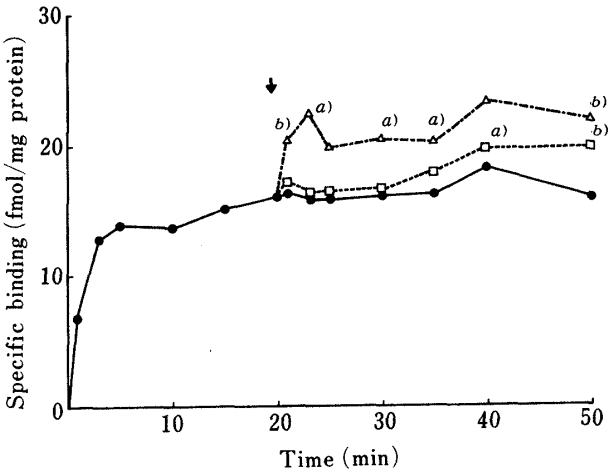

Fig. 3. Effects of Choline and Inositol on β-Adrenoceptor Binding

The guinea pig cerebral cortical membranes were incubated at 23 °C for various periods with 1.2 nM ^{3}H-DHA in the absence (●) and presence of choline (□) and of inositol (△). Choline and inositol (1 μM) were added to the incubation mixtures at the times (after 20 min of incubation) indicated by the arrow. The data represent the mean values ± S.E. of duplicate determinations from four separate experiments. Paired Student's *t*-test: *a*) $p<0.05$ or *b*) $p<0.02$ *vs.* without choline and inositol.

TABLE III. Effects of Removal of Choline and Inositol

Pre-incubation Choline (1 μM)	Pre-incubation Inositol (1 μM)	Incubation Choline (1 μM)	Incubation Inositol (1 μM)	*n*	Specific binding (fmol/mg protein)
−	−	−	−	3	13.73 ± 0.68
−	+	−	−	3	19.10 ± 1.29[a)]
−	+	−	+	3	18.15 ± 1.16[a)]
+	−	−	−	3	20.48 ± 1.16[a)]
+	−	+	−	3	20.00 ± 1.98[a)]

Values are means ± S.E. The values (*n*) are the numbers of experiments. Student's *t*-test, *a*) $p<0.05$ *vs.* control in the absence of choline and inositol. The pre-incubation of the guinea pig brain membranes (10 mg of protein) in 10 ml of 60 mM Tris–HCl, 20 mM $MgCl_2$ buffer (pH 7.4) was carried out at 23 °C for 30 min in the absence and presence of choline and inositol (1 μM) and then the membranes were centrifuged at 40000 *g* for 30 min. The pellets were rinsed at once with 120 mM Tris–HCl, 40 mM $MgCl_2$ buffer (pH 7.4) and then suspended in 10 ml of the same buffer. The membranes were further centrifuged at 40000 *g* for 30 min, rinsed, and suspended twice more. The incubation of the membranes with 1.2 nM ^{3}H-DHA was carried out at 23 °C for 30 min in the absence and presence of choline and inositol (1 μM) using the binding assay method described in the text.

TABLE IV. Effects of Choline and Inositol on the Specific Binding of ^{3}H-DHA to Guinea Pig Brain Membrane

	n	B_{max} (fmol/mg protein)	K_d (nM)	Hill coefficient
Control	(4)	60.59 ± 3.55	4.14 ± 1.00	0.99 ± 0.02
Choline	(4)	86.53 ± 7.74[a)]	5.22 ± 1.17	0.98 ± 0.01
Inositol	(4)	89.59 ± 9.35[a)]	3.98 ± 0.70	0.97 ± 0.01

The data were obtained by Scatchard analysis. Values are means ± S.E. Numbers in parentheses represent the numbers of experiments. Student's *t*-test, *a*) $p<0.05$ *vs.* control.

and inositol on β-adrenoceptor binding were examined in this study in order to characterize the activation factors in Eagle's MEM.

Figures 1 and 2 show the influence of the concentrations of choline and inositol on the specific binding. The addition of 0.1 to 10 μM choline and inositol caused an increase in the specific binding. These results suggest that choline and inositol show optimal concentrations for the activation of the specific binding. Therefore, the experiments mentioned below in the

TABLE V. Effects of Choline and Inositol on the Specific Binding of ^{125}I-ICYP to Guinea Pig Brain Membranes

	Low-affinity site		High-affinity site		Total	Low : high ratio	Hill coefficient
	K_d (pM)	B_{max} (fmol/mg protein)	K_d (pM)	B_{max} (fmol/mg protein)	B_{max}		
Control (4)	526.44±102.69	70.54±3.59	8.81±0.91	3.88±1.41	74.42±3.59	94.8 : 5.2	0.86±0.06
Choline (4)	679.41± 65.11	94.74±7.37[a)]	12.41±4.35	3.93±1.12	98.67±8.14	96.0 : 4.0	0.85±0.04
Inositol (4)	683.45± 41.90	97.00±4.75[b)]	14.82±5.08	5.03±1.49	102.03±4.10	95.1 : 4.9	0.83±0.04

Values are means±S.E. Numbers in parenthesis represent the number of experiment. Student's *t*-test, *a*) $p<0.05$ *vs.* control. *b*) $p<0.02$ *vs.* control.

text were carried out with the concentration of 1 μM choline and inositol.

As shown in Fig. 3, the effects of choline and inositol were observed at 20 and 1 min after the addition of choline and inositol, respectively and the effects were maintained at 30 min after the addition.

The effects of removal of choline and inositol on the specific binding were examined. As shown in Table III, choline and inositol significantly increased the values of the specific binding even when these chemicals were washed out of the membranes after pre-incubation.

Tables IV and V summarize the results on the addition of choline and inositol to the β-adrenoceptors obtained by Scatchard analysis using ^{3}H-DHA or ^{125}I-ICYP as radioligands. Choline and inositol significantly increased (42.8 and 47.9%, respectively) the density of β-adrenoceptors but no significant difference was observed in the values of the dissociation constant (K_d) when ^{3}H-DHA was used as a radioligand (Table IV). In the ^{125}I-ICYP binding, choline and inositol significantly increased (34.3 and 37.5%, respectively) the capacity of the binding sites (B_{max}) of the low-affinity sites (Table V). The values of the Hill coefficient obtained by the addition of choline and inositol were constant as compared with control values in the absence of choline and inositol (Tables IV and V).

Discussion

In this study, we showed that choline and inositol in Eagle's MEM significantly increased the density of the β-adrenoceptors of guinea pig cerebral cortical membranes. The increases were obtained after incubation for 20 min in the case of choline and 1 min in the case of inositol and were maintained at 30 min. The removal of these chemicals after pre-incubation had no effect on the increase of the binding sites. These results suggest that choline and inositol irreversibly changed the membrane function related to β-adrenoceptors.

It is known that changes of the membrane function and structure cause changes of the binding sites and/or the affinities of β-adrenoceptors. Other investigators reported[7)] that the addition of phospholipids to membrane-bound and solubilized β-adrenoceptors of rabbit heart caused an increase in the B_{max}, suggesting that the phospholipids stabilized the membranes and increased the number of the binding sites. On the other hand, adenylate cyclase coupled to β-adrenoceptors was activated by the addition of some fatty acids, such as palmitoleic, oleic, *cis*-vaccenic and eicosenoic acid[8)] and inhibited by the addition of lysolecithin[9)] and cholesterol.[10)] As is well known, these chemicals, phospholipids, fatty acid, lysolecithin and cholesterol, are membrane components. The dependence of the rate of adenylate cyclase activation by a hormone receptor on membrane fluidity can be adopted as a general diagnostic parameter for investigating coupling between a hormone receptor and adenylate cyclase.[11)] The changes of the membrane fluidity by *l*-isoproterenol, a β-adrenoceptor agonist, also caused an increase in phospholipid methylation.[12)] These observations raised

the possibility that the addition of phospholipid caused an increase in the number of binding sites or affinity of β-adrenoceptors. It is interesting to note that choline and inositol are membrane components and are constituents of phospholipids.

We reported previously that the addition of anionic and cationic polymeric effectors, such as polymyxin B, heparin, and DNA, caused a reduction in the number of binding sites and/or an increase in the affinities of β-adrenoceptors,[1] that the addition of lectins caused an increase in the number of binding sites and the affinities, [2a] and that the treatment of the membranes with neuraminidase, endoglycosidase H, and glycopeptidase A caused a reduction in the number of binding sites and/or an increase in the affinities of β-adrenoceptors.[2b] As described above. phospholipids, choline and inositol increased the number of binding sites but did not change the affinities of β-adrenoceptors. These results imply that changes of the membrane function and structure cause widely various effects on the binding sites and affinities of β-adrenoceptors.

The signalling system using cyclic adenosine monophosphate (c-AMP) as the second messenger may be the best known, and the β-adrenoceptors conform to this system. Another major signalling pathway utilizes the inositol lipids as part of the transduction mechanism, and the muscarinic and α_1-adrenergic receptors conform to this inositol phosphate cycle.[13] Hokin and Hokin reported[14] that acetylcholine stimulated the exchange of phosphate in phosphatidic acid in the guinea pig brain and the stimulation was inhibited by atropine. Furthermore, Friedel *et al.* reported[15] that phenylephrine enhanced the labeling of phosphatidic acid and phosphatidyl inositol in the rat brain, and the stimulation was inhibited by phenoxybenzamine. However, isoproterenol, which is a β-adrenoceptor agonist, had no effect on the labeling of phosphatidic acid and phosphatidyl inositol,[15] suggesting that the metabolism of phosphatidic acid and phosphatidyl inositol in the brain of guinea pig and rat is associated with a rapid membrane process controlled in part by muscarinic and α_1-adrenergic mechanisms, differing from the effects which are produced by the β-adrenergic mechanism. Inositol is the end product of the inositol phosphate cycle. Therefore, it is of interest to note that inositol causes an increase the β-adrenoceptor binding sites (this study), and the β-adrenoceptors may be related to the signalling system of other receptors.

We have reported that the Scatchard plots of ^{3}H-DHA binding show uniphasic character, suggesting that this radioligand was non-selective for β_1- and β_2-adrenoceptors.[5a,b] Therefore, we used this ligand for the determination of the changes of whole β-adrenoceptors in this study. We also reported that the Scatchard plots using ^{125}I-ICYP show biphasic character, suggesting that high- and low-affinity sites were identical to β_2- and β_1-adrenoceptors.[5b,c] These results imply that the values of K_d and B_{max} of β_1- and β_2-adrenoceptors can be directly determined by analyzing the binding behavior of ^{125}I-ICYP (biphasic Scatchard plots). In the present study, choline and inositol caused an increase in the B_{max} value of β_1-adrenoceptors, but not β_2-adrenoceptors. It seems to be important that these chemicals have an influence only on β_1-adrenoceptors, because β_1- and β_2-adrenoceptors have been suggested[16] to have different structures or amino acid sequences.

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