Biochemical Pharmacology, Vol. 56, pp. 237–241, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00112-9

Region-specific Changes in CNS Muscarinic Acetylcholine Receptors in a Rat Model of Hyperammonemia

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ABSTRACT. Multiple neurotransmitter systems have been implicated in the etiology of cerebral dysfunction in acute and chronic hyperammonemic states. Involvement of the neurotransmitter systems of glutamate and γ-aminobutyric acid has been reported, whereas not much information is available on the role of the cholinergic system in the etiology of hyperammonemic states. In the present investigation, muscarinic acetylcholine receptors (mAChR) were studied, using tritium-labelled quinuclidinyl benzilate ([^{3}H]QNB), in rats administered ammonium acetate (AA), and the changes in the treated rats were compared with measurements in normal rats. The presence of two affinity (high and low) systems for [^{3}H]QNB binding was observed in the cerebral cortex (CC), while a single affinity system was seen in the cerebellum (CE) and pons-medulla (PM). A decrease in the B_{max} of both the high and low affinity systems for [^{3}H]QNB binding was observed in the CC with no significant change in the K_d values in rats administered an acute dose of AA (25 mmol/kg of body weight). The B_{max} values were unaltered in CE and PM, but a significant increase in the K_d value was observed in the CE. Studies of [^{3}H]QNB binding in the presence of pirenzepine (a specific antagonist of M_1 receptors) indicated the predominance of non-M_1-type (M_2, M_3, M_4, and M_5) receptors (85–90% of the total specific binding) in the CE and PM, whereas in the CC, 60% was represented by non-M_1 and 40% by M_1 receptors. Reduction in [^{3}H]QNB binding to M_1 receptors was observed in the CC and PM of rats administered an acute dose of AA. Administration of lower doses of AA (2.5 mmol/kg of body weight) had no effect on CC and CE mAChRs, while an increase in non-M_1 and a decrease in M_1 receptors was observed in the PM. As the neurotransmitter receptors play a key role in signal transduction, the observed changes in receptor functions may be responsible for some of the behavioral changes reported in hyperammonemic states. BIOCHEM PHARMACOL **56**;2:237–241, 1998.

KEY WORDS. CNS; hyperammonemia; muscarinic acetylcholine receptors

Hyperammonemia prevails in many liver diseases such as fulminant hepatic failure, cirrhosis, and necrosis of the liver and in inborn errors of urea cycle enzymes. All of these disorders are associated with abnormal functioning of the brain, and the individuals exhibit a wide spectrum of neurobehavioral changes, called "hepatic encephalopathy." At pathophysiological concentrations, ammonia is known to interfere with metabolic and neurotransmitter functions of the brain, leading to coma and death [1–3]. Involvement of the glutamatergic system has been well studied in acute and chronic hyperammonemia, and it has been reported that ammonia at pathological concentrations exerts differential effects on the subtypes of glutamate and GABA† receptors [2]. The cholinergic system is known to play a key role in the neuronal mechanisms underlying learning and memory, thought processing, attention, sleep–wake cycles, and control of movement, which are reported to be altered during hyperammonemic states [2–7]. Hence, in the present study, experiments were carried out on mAChRs, which play an important role in signal transduction of the cholinergic system.

MATERIALS AND METHODS

Adult Wistar rats weighing 200–240 g were used as experimental animals. [^{3}H]QNB (43.5 Ci/mmol) was purchased from DuPont/NEN Products. All other chemicals were purchased from local companies.

Hyperammonemia was induced, as described by Rukmini Devi and Murthy [8], by intraperitoneal administration of a single dose of AA (acute group: 25 mmol/kg of body weight; subacute group: 2.5 mmol/kg of body weight), and the rats were decapitated after 20 min. Membranes were prepared from the CC, CE, and PM of normal and hyperammonemic rats by the method of Rao *et al.* [9].

Receptor binding studies were performed as described by

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† *Abbreviations:* AA, ammonium acetate; CC, cerebral cortex; CE, cerebellum; GABA, γ-aminobutyric acid; mAChR, muscarinic acetylcholine receptor; PM, pons-medulla; and [^{3}H]QNB, tritium-labelled quinuclidinyl benzilate.

Received 5 August 1997; accepted 25 February 1998.

Rao *et al.*, [9]. Membrane preparation (200 μg of protein equivalent) was incubated with [^{3}H]QNB (0.005 to 2 nM for kinetic studies and 1 nM for routine assays) in 500 μL of 40 mM of Tris–HCl buffer (pH 7.4) for 15 min at 37°. Nonspecific binding was determined in parallel assays performed in the presence of 100 μM of atropine. To study the subtypes of muscarinic receptors, M_1 receptors were blocked by incubating the membranes with 50 nM of pirenzepine (for 20 min) prior to the addition of 1 nM of [^{3}H]QNB. [^{3}H]QNB bound under these conditions was considered as non-M_1 binding. The difference between binding in the absence and in the presence of pirenzepine was considered as the [^{3}H]QNB bound to M_1 receptors. In all of these studies, binding was stopped by diluting the reaction mixture with 6 mL of ice-cold Tris–HCl buffer (pH 7.4) and rapid filtration under vacuum through GF/C glass microfiber filters, followed by two washes with ice-cold Tris–HCl buffer. Filters were dried and transferred to scintillation vials containing 5 mL of Bray's mixture, and radioactivity was determined in a Beckman LS-1800 liquid scintillation spectrometer at 55% efficiency. To reduce the nonspecific binding of the ligand, filter discs were soaked in 0.1% (v/v) polyethylenimine for 1 hr prior to use.

Kinetic constants for [^{3}H]QNB binding were calculated by Scatchard analysis of the data. Statistical significance between two groups was calculated by Student's *t*-test and statistical comparisons between multiple groups were done by one-way ANOVA (post test: Student–Newman–Keuls test) using the Sigmastat program.

RESULTS

A sequence of behavioral changes was observed following the administration of the acute dose of AA. Five minutes after AA administration, animals became lethargic and lost their sense of equilibrium and their posture. Convulsions were observed between 25 and 30 min after AA administration, and the mortality rate was very high at this stage. Hence, the animals were killed 20 min after the administration of the drug, i.e. before they entered into the convulsive phase. No such changes were observed in rats administered the subacute dose of AA, and these animals completely returned to normal.

All preliminary standardization experiments were performed with membranes prepared from the CC of control rats. [^{3}H]QNB binding was saturable and reversible. In all these studies, nonspecific binding was less than 5% of total [^{3}H]QNB binding. The reversibility of [^{3}H]QNB binding was verified by the addition of unlabelled atropine (an antagonist of muscarinic receptors), after incubating the membranes with [^{3}H]QNB (data not shown).

Regional differences were observed in the distribution of muscarinic receptors in brain. [^{3}H]QNB binding was highest in the CC (1.23 ± 0.13 pmol/mg of protein) followed by the PM (0.371 ± 0.046 pmol/mg of protein) and the CE (0.189 ± 0.026 pmol/mg of protein). Regional differences were also observed in the distribution of muscarinic receptor subtypes. The density of both M_1 and non-M_1 (M_2, M_3, M_4, and M_5) receptors was higher in the CC than in the CE and PM. The lowest density was observed in the CE. Differences were also observed in the relative contribution of M_1 receptors to the total population of receptors in these three regions. In the CC, 40% of the mAChRs were contributed by M_1, whereas in the CE and PM it was 10–15%. The rest of the receptor population might be represented by other subtypes of mAChRs, considered as non-M_1 (Fig. 1).

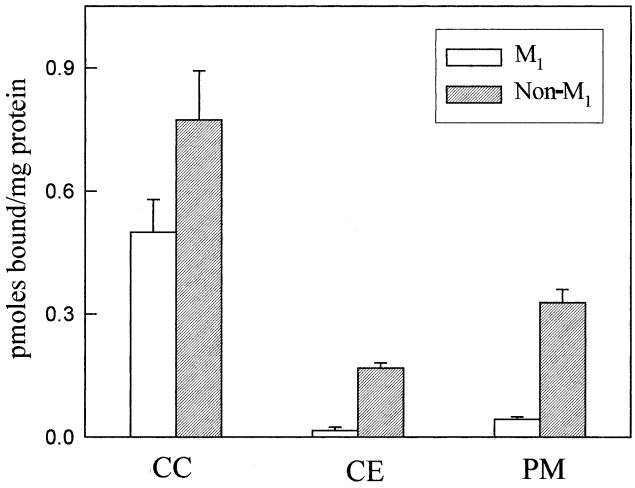

FIG. 1. Regional distribution of subtypes of mAChRs (non-M_1 and M_1) in the CC, CE, and PM. Membranes were incubated with 1 nM of [^{3}H]QNB in the absence (total) and in the presence (non-M_1) of pirenzepine (a specific antagonist of M_1 receptors). The difference between total and non-M_1 was considered to be M_1 binding. Values are means ± SD of four experiments done in duplicate.

Regional differences were observed in saturation isotherms for [^{3}H]QNB binding in the brain. In the CE and PM, saturation isotherms were monophasic (Fig. 2). However, the saturation isotherm for [^{3}H]QNB binding in the CC was observed to be biphasic (Fig. 3). The first phase of [^{3}H]QNB binding reached saturation at 0.05 nM. An increase in [^{3}H]QNB concentration beyond 0.05 nM resulted in a steep increase in [^{3}H]QNB binding, and the second phase of saturation was observed at 0.5 nM (Fig. 3). The data for [^{3}H]QNB binding in the CE and PM could be fitted into a single linear curve with first order regression in Eadie–Scatchard plots (Fig. 2, insets). In contrast, data from the CC gave a curvilinear Scatchard plot, which fits best into a biaffinity system—a high-affinity binding system (HABS) and a low-affinity binding system (LABS; Fig. 3, inset). Accordingly, two K_d and two B_{max} values were calculated for [^{3}H]QNB binding in the CC. K_d and B_{max} values for LABS were 3- to 4-fold higher than that of HABS (Table 1).

Studies in hyperammonemic rats revealed no significant alterations in [^{3}H]QNB binding in the CE in either the acute or subacute group. A significant decrease in [^{3}H]QNB binding was observed in the CC of the acute group while a significant increase was observed in the PM of the subacute

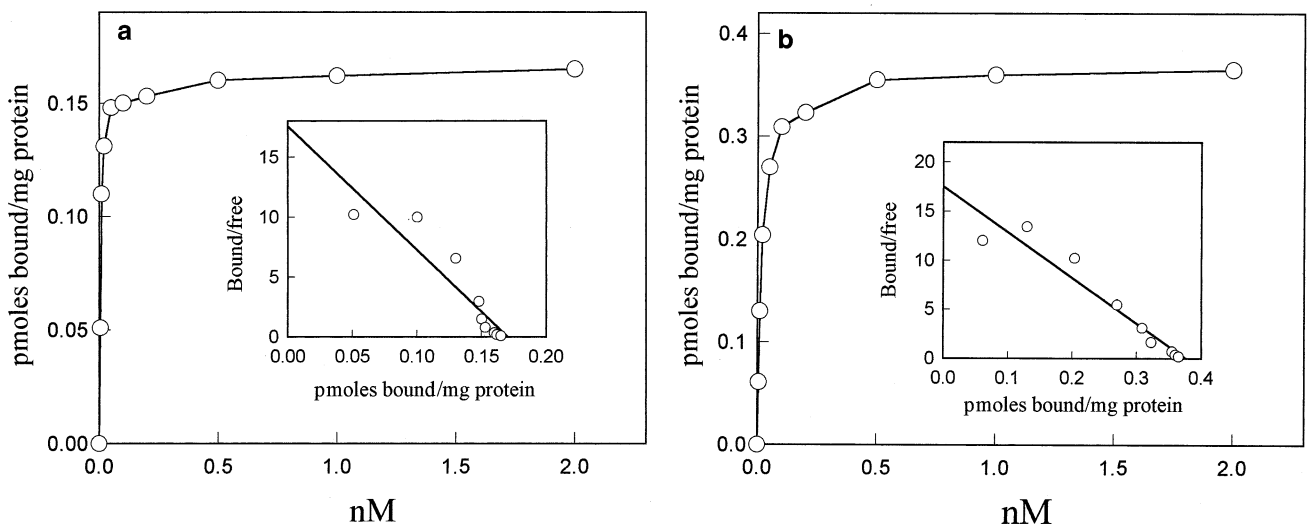

FIG. 2. Saturation isotherms for [^{3}H]QNB binding in (a) CE and (b) PM, showing a single affinity system. Insets: Scatchard plots of the data. Values are means of four experiments done in duplicate.

group. Regional differences were observed in the response of M_1 and non-M_1 subtypes of mAChRs to hyperammonemic states. The binding of QNB to M_1 receptors was suppressed in both the CC and PM in acute hyperammonemic states, whereas in the subacute state a significant decrease was observed only in the PM. There was no significant change in [^{3}H]QNB binding to M_1 receptors of the CE under these conditions. [^{3}H]QNB binding to non-M_1 receptors was elevated in the PM of subacute rats, while there were no changes in the other two regions in both acute and subacute states (Fig. 4, a–c).

Kinetic studies of [^{3}H]QNB binding in acute hyperammonemic rats indicated a decrease in B_{max} values of high and low affinity [^{3}H]QNB binding systems in the CC while there were no significant changes in the B_{max} values of [^{3}H]QNB binding in the CE and PM. There were no significant changes in the K_d values for [^{3}H]QNB binding in the CC and PM, whereas a significant increase in the K_d value was observed in the CE (Tables 1 and 2). AA added *in vitro* (to membranes isolated from normal rats) had no significant effect on [^{3}H]QNB binding.

DISCUSSION

An interesting observation in this study was the presence of two affinity systems for [^{3}H]QNB binding in the CC. Kloog and Sokolovsky [10] reported the apparent presence of two types of binding sites in mouse brain membranes for *N*-methyl-4-piperidyl benzilate (4-NMPB), an analogue of the benzilate series, and for scopolamine (a muscarinic antagonist). They proposed that this was due to the formation of receptor–ligand complexes with different dissociation constants. However, with the data available, it is not possible to predict whether the dual affinity systems

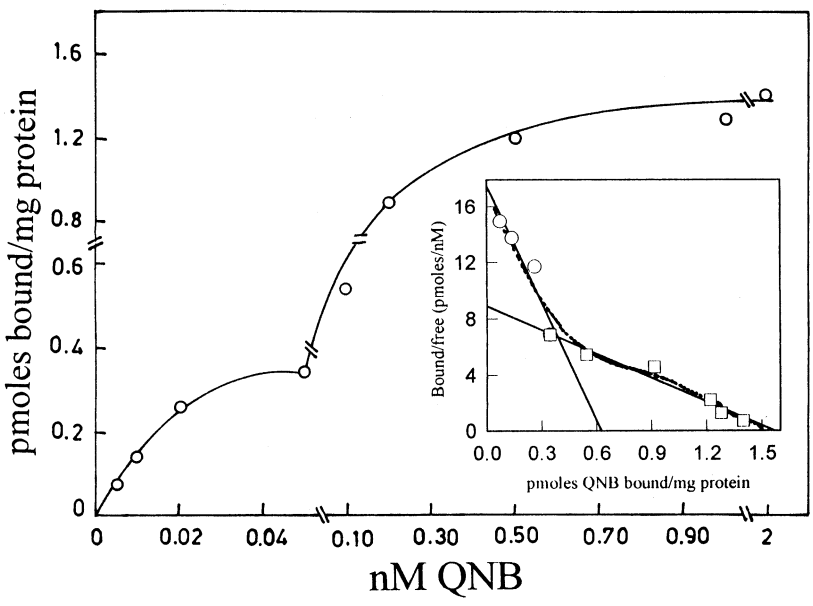

FIG. 3. Saturation isotherm showing the biphasic nature of [^{3}H]QNB binding to cortical synaptic membranes. Inset: Scatchard plot for QNB binding showing the possible presence of two affinity systems for [^{3}H]QNB binding in the CC. Values are means of four experiments done in duplicate.

TABLE 1. Kinetic constants for [^{3}H]QNB binding in synaptic membranes isolated from the CC of control rats and rats administered an acute dose of AA

	High affinity		Low affinity	
Group	K_d	B_{max}	K_d	B_{max}
Control	42 ± 9	0.62 ± 0.07	189 ± 14	1.68 ± 0.31
Acute	35 ± 7	0.47 ± 0.07*	213 ± 21	1.01 ± 0.14†

Values are means ± SD of four experiments done in duplicate. K_d values are expressed in pM, and B_{max} values are expressed as pmol of QNB bound/mg of protein.

*Significantly different from control, $P = 0.01$.

†Significantly different from control, $P = 0.003$.

observed in the present study actually represent two isoforms of a single receptor–ligand complex (as suggested by Kloog and Sokolovsky) or two distinct types of muscarinic receptors.

The decrease in B_{max} of [^{3}H]QNB binding to the CC in hyperammonemic states suggested a decrease in mAChR density in the CC. Studies on receptor subtypes using pirenzepine indicated that the M_1 and non-M_1 receptors are differentially distributed among the regions studied. This is in agreement with results published earlier [11]. A decrease in QNB binding to M_1 receptors in the CC and PM but not in the CE suggested that pathological concentrations of ammonia exert differential effects in different regions of the brain. Similar changes in muscarinic M_1 and M_2 subtypes have been reported in congenital ornithine transcarbamylase deficient mice, an animal model of chronic hyperammonemia [12]. The lack of effect of AA on [^{3}H]QNB binding under *in vitro* conditions suggests that ammonia may not be interacting with the receptor directly. Hence, the observed changes in receptor densities in hyperammonemic states may be attributed to altered turnover rates (synthesis and degradation) of the receptor protein or be due to changes in membrane fluidity and local environment of the receptor protein. The first possibility can be ruled out, as the time lapsed between administration of AA and killing of the animal was too short to account for the altered rates of synthesis and degradation of the receptor protein. Hence, the observed changes in receptor densities during *in vivo* hyperammonemic states might be through some other processes such as changes in membrane fluidity or local environment of the receptor, as suggested earlier [2].

A decrease in M_1 receptor density during hyperammonemia might affect the functions mediated by these receptors. It has been reported that the cholinergic neurons in the brain are involved in thought processing, initiation and execution of motor functions, and in the regulation of sleep–wake cycles [7]. Defects in motor functions such as loss of equilibrium, involuntary movements, confusion, and EEG changes have been reported in patients with hepatic encephalopathy and hyperammonemia and also in animal models of hyperammonemia, which are known to be controlled by the cholinergic system [2]. As M_1 receptors

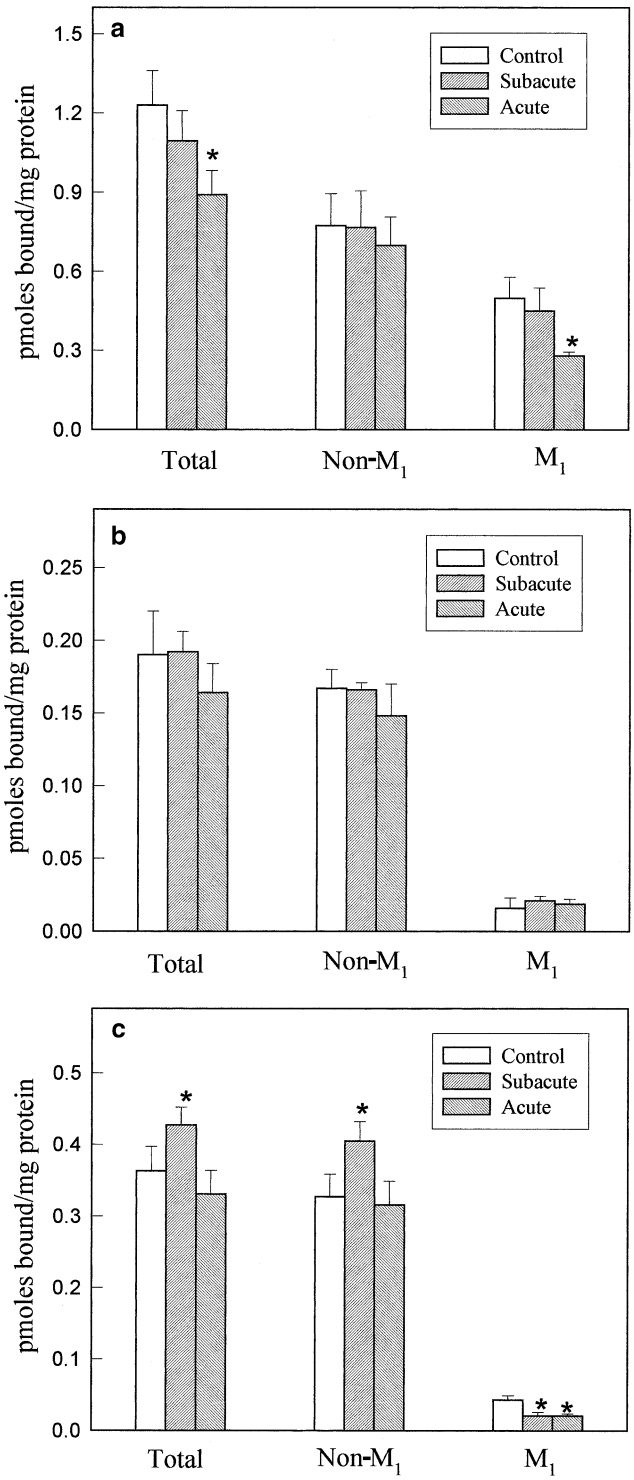

FIG. 4. Specific [^{3}H]QNB binding to total, non-M_1, and M_1 muscarinic receptors in the (a) CC, (b) CE, and (c) PM of control rats and rats administered a subacute or an acute dose of AA. Values are means ± SD of four experiments done in duplicate. *Statistically significant compared with controls, $P < 0.01$.

are post-synaptic in localization and play a crucial role in signal transduction, impaired function of these receptors in hyperammonemic states may be responsible for some of the behavioral changes reported in hyperammonemic states.

TABLE 2. Kinetic constants for [^{3}H]QNB binding in synaptic membranes isolated from the CE and PM of control rats and rats administered an acute dose of AA

	Control		Acute	
	K_d	B_{max}	K_d	B_{max}
CE	9.0 ± 1.6	0.17 ± 0.03	12 ± 1.4*	0.19 ± 0.05
PM	28 ± 5	0.42 ± 0.03	23 ± 3.5	0.37 ± 0.04

K_d values are expressed in pM, and B_{max} values are expressed as pmol of ligand bound/mg protein. Values are means ± SD of four experiments done in duplicate.
*Significantly different from control, $P = 0.01$.

It is well established that multiple neurotransmitter systems are involved in the pathogenesis of hepatic encephalopathy and hyperammonemia [13]. Hyperammonemia interferes with both metabolism and neurotransmitter functions of glutamate such as release, uptake, and binding [2]. Recent evidence suggests that ammonia toxicity is mediated by *N*-methyl-D-aspartate (NMDA) receptors, and administration of MK-801, an antagonist of NMDA receptors, attenuates the neurotoxic effects of ammonia to some extent [14]. A selective loss of post-synaptic D_2 receptors was observed in patients with portal systemic encephalopathy [15]. Changes in GABA receptors have also been reported in hyperammonemia [9]. A report in human subjects with hepatic encephalopathy suggests no changes in activities of the enzymes choline acetyltransferase and acetylcholinesterase [16]. However, recent studies on cholinergic function in hyperammonemia indicated a decrease in choline acetyltransferase activity, an increase in acetylcholinesterase activity, and a loss of M_1 receptors [8, 12, 17]. These changes together suggest a hypofunction of the cholinergic system in hyperammonemia, which supports clinical findings such as confusion and abnormal motor activity observed in patients with hepatic encephalopathy and hyperammonemia. Ammonia also exerts a direct effect on neuronal membranes. At low concentrations, ammonia abolishes the post-synaptic inhibitory potential, and at concentrations >1 mM of ammonia depolarizes the neuronal membrane, thus making the neurons more excitable. This could be the reason for the observed seizures in ammonia intoxication, which are exclusively spinal in origin and not of cortical origin [18]. Although the animals were killed prior to the onset of seizures, pre-seizure electrical activity cannot be ruled out. This might also contribute to the changes observed in the present study. All of these findings indicate that the pathogenesis of hyperammonemia involves multiple neurotransmitter systems. However, it is yet to be established whether the changes in all the neurotransmitter systems contribute synergistically to the clinical findings or if only one neurotransmitter system is mainly involved and the other systems are involved in further potentiating the symptoms.

Thanks to Dr. V. L. R. Rao for his suggestions in preparing the manuscript. This work was supported by grants from the Indian Council of Medical Research. R. P. Rukmini Devi received a fellowship from the Council of Scientific and Industrial Research, India.

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