The change of picrotoxin-induced epileptiform discharges to the beta oscillation by carbachol in rat hippocampal slices

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The study aimed to determine whether and how the activation of the acetylcholine receptor affects epileptiform discharges in the CA3 region in a rat hippocampus. Picrotoxin (100 μM), a GABA_A receptor antagonist, was applied to a hippocampal slice to induce epileptiform discharges. The effects of the cholinergic agonist, carbachol, on the discharges were examined at the several concentrations (1–30 μM). Carbachol had different impacts on epileptiform discharges at the different concentrations. Relatively low concentrations of carbachol (<10 μM) increased the frequency but decreased the amplitude of the discharges. At 10 μM, carbachol induced the discharges, including bursts of theta frequency oscillations. At 30 μM, carbachol could induce bursts of beta frequency oscillations instead of epileptiform discharges. The amplitudes of the oscillations were smaller than those of the discharges. Carbachol suppressed the evoked population EPSPs (pEPSPs) in a dose-dependent manner. These effects were blocked by the muscarinic cholinergic receptor antagonist atropine sulfate. The high level of muscarinic receptor activation can replace epileptiform discharges with theta or beta oscillation. These results suggest that the dose-dependent alternation of the acetylcholine receptor activation may provide the three different stages the epileptiform discharges, the bursts of theta oscillation, and the bursts of the beta oscillation.

Key words: epileptiform discharges, carbachol, beta oscillation, desynchronization, population excitatory postsynaptic potential

Acetylcholine (ACh), an important neurotransmitter, is involved in multiple brain functions such as arousal, attention, learning, and memory [1–4]. Cholinergic neurons project to widespread areas in the central nervous system including the hippocampus. The medial septal nucleus predominantly releases ACh to the hippocampus through septo-hippocampal cholinergic projections [5,6]. The cholinergic system contributes to synchronized neuronal oscillations in different frequency ranges, including the theta (4–12 Hz), beta (13–30 Hz), and gamma (>30 Hz) frequencies in the rat hippocampus [7–10]. These synchronized oscillations are thought to contribute to the memory process [8,11–15]. The cholinergic system also has been intensively studied in pathological conditions such as epilepsy [16]. Epilepsy is a disease characterized by recurrent seizures [17]. An epileptic seizure is a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain, accompanied by abnormal electroencephalogram activations with large amplitudes. The massive neuronal synchronization firing is thought to be important for the generation of the seizures [18,19]. The hippocampus is the most common site for epileptic foci [20]. An understanding of the relationship between the cholinergic system and neuronal synchronization in the hippocampus in both physi-

A cholinergic agent, carbachol, altered the induction patterns of the picrotoxin-induced epileptiform discharges in a concentration-dependent manner. At the lower concentration it would suppress the synchronous population firing of the pyramidal cells, and in results, carbachol decreased the amplitude of the discharges and increased the frequency. At the higher concentration, it induced theta or beta oscillations instead of the epileptiform discharges. Not only hippocampal theta rhythm but also hippocampal beta rhythm would suppress the epileptiform discharges.
The modulation of the epileptiform discharges and the ACh-induced reduction in the pEPSP slope induced by carbachol may affect the induction of epileptiform discharges. In addition, epileptic seizures preferentially occur during slow-wave phases of sleep, and are less frequent during wakefulness and rapid eye movement (REM) sleep [23,24]. The ACh levels in the hippocampus are high during slow-wave sleep and REM sleep, and drop to a minimum during slow-wave sleep [25,26]. Thus, it is possible that the activation level of the cholinergic receptor in the hippocampus may affect the induction of epileptiform discharges. In addition, septo-hippocampal projection contributes to generate hippocampal theta rhythm. The projections will also contribute to generate hippocampal beta rhythm [9]. The relationship between cholinergic receptor activation level in the hippocampus and the induction of epilepsy is still under investigation.

The cholinergic agonist, carbachol, has been observed to induce several kinds of oscillations in hippocampal slices [27]. The synchronization behavior of hippocampal neuronal networks is modulated by the frequency of neuronal oscillations [27,28]. Picrotoxin, a GABA_A receptor antagonist can induce epileptiform discharges in hippocampal slices [29].

To examine the effect of cholinergic receptor activation on epileptiform discharges, we evaluated the effect of several concentrations of carbachol on the picrotoxin-induced epileptiform discharges induced in a hippocampal slice preparation. In the present study, we show that the activation of the muscarinic cholinergic receptor has differential impact on synchronous epileptiform discharges depending on the level of the activation. At lower activation levels, the epileptiform discharges had smaller amplitudes and higher frequencies. At higher levels of the activation of the ACh receptor, theta or beta frequency oscillations began to be induced, and eventually suppressed the epileptiform discharges. We also show the ACh-induced reduction in the slope of the evoked population excitatory postsynaptic potentials (pEPSP) in recurrent connections of the CA3 pyramidal cells. The modulation of the epileptiform discharges and the reduction in the pEPSP slope induced by carbachol may contribute to the cholinergic modulation of epileptiform discharges.

Materials and Methods

Experimental animals and hippocampal slice preparations

The data were obtained from 46 hippocampal slices from 27 male Wistar rats aged 3–5 weeks. The experimental procedures used in this study were approved by the Committee for the Animal Care and Use of Laboratory Animals at the Graduate School of Life Science and System Engineering of Kyushu Institute of Technology. The approval number of the experiment is Sei-10-13. The experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals at the Graduate School of Life Science and System Engineering of Kyushu Institute of Technology. The rats were anesthetized with diethyl ether and decapitated. The brains were dissected and placed in ice-cold artificial cerebrospinal fluid (ACSF) solution with the following composition (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 26 NaHCO₃, 10 glucose, and 2 CaCl₂. Transverse 450-μm-thick hippocampal slices were prepared using a tissue slicer (Micro Slicer Zero-1, Dosaka-EM Co., Japan). The slices were transferred to an interface recording chamber and incubated in ACSF bubbled with a 95% O₂-5% CO₂ solution at 33.5±1°C for at least 1 h for recovery from dissection. The slices on the chamber were continuously perfused with ACSF at a flow rate of 1.5 ml/min.

Recording and stimulation

Extracellular field potentials were recorded from the CA3 region in the hippocampal slices using a glass microelectrode filled with 2 M NaCl (~2.5 MΩ). Spontaneous field potentials, picrotoxin-induced epileptiform discharges, and carbachol-induced theta and beta oscillations were recorded from the stratum pyramidale. Evoked pEPSPs were recorded from the stratum radiatum following stimulation of the Schaffer collaterals. A concentric polar stimulation electrode was placed in the stratum radiatum of the CA3 region. The stimulation intensity was adjusted so as to evoke a pEPSP with amplitude of 50–60% of the maximum amplitude. The actual stimulation intensity was set at 60–300 μA, and the duration of the stimulation pulse was 100 μs. The stimulation was delivered every 60 s during the last 6 min before the application of carbachol, and at the perfusion of each concentration of carbachol; carbachol was applied for 20 min at each concentration. The field potentials recorded from the glass microelectrodes were amplified (~1000) and filtered with low-pass (0.3 kHz) and high-pass (1 Hz) filters using an ER-1 extracellular amplifier (CYGNUS Technology Inc., Southport, NC). For the pEPSP recordings, a low-pass filter of 30 kHz and a high-pass filter of 1 Hz were used. The sampling frequency was set at 1 kHz for recording epileptiform discharges, theta and beta oscillations, and 10 kHz for
the pEPSP recordings. The signals were sampled and stored on a computer using the pClamp10.0 software (Molecular devices Co., Sunnyvale, CA).

Pharmacological manipulation

The hippocampal slices were constantly bathed in picrotoxin (100 μM), to induce epileptiform discharge. The cholinergic agonist, carbachol, was applied after 30 min of picrotoxin application, while the epileptiform discharges were in a steady state. Furthermore, carbachol concentrations of 1, 5, 10, and 30 μM were each added for 20 min. Selective cholinergic antagonists were applied 10 min before the application of carbachol. All the drugs were purchased from Sigma-Aldrich Japan Co., Japan.

Data analysis

For the analysis of epileptiform discharges, the frequency was calculated from the inter-discharge interval, and the peak-to-peak amplitude was measured. Carbachol (>10 μM) induced prolonged field potential oscillations with durations of about 10 s, separated by regular intervals. Four parameters were analyzed for the burst as described below: frequency, amplitude, duration, and interburst interval. The frequency within each oscillation changed over time, and remained stable for 1–2 s after 2 s of the onset of the oscillation. The peak frequency within an individual oscillation was measured by Fast Fourier Transform analysis for 1 s during which the frequency was stable. The peak-to-peak amplitudes of the oscillation were measured for the same period to analyze the amplitude. We measured the time from the start to the end of each burst as a duration. We measured the time between the end of one burst and the start of the next. The time was defined as an interburst interval. There are several definitions for the interval. We have adopted the definition used in electroencephalography study field [30].

The initial slopes of the pEPSPs were measured using the data at the open arrow in Figure 2 for 1 ms. We used the least squares approximation to calculate the slope.

The relative values were defined as the ratio with the averaged data obtained after the application of carbachol to the data before it. One-way ANOVA followed by a post-hoc Scheffe test or a Fischer’s test was used to test statistical significance. The statistical significance threshold was set at p<0.05. The data were expressed as means±standard errors of the mean (S.E.M).

Results

Effects of the cholinergic agonist on picrotoxin-induced epileptiform discharges

The application of picrotoxin (100 μM) induced interictal-like epileptiform discharges in the hippocampal slices, as previously reported [29]. The average frequency, amplitude, and duration of the epileptiform discharges were 0.06±0.01 Hz, 5.00±0.37 mV, and 0.20±0.01 sec, respectively (n=5) (Fig. 1A, top trace). To determine whether cholinergic receptor activation affects the properties of the epileptiform discharges, we applied carbachol to the hippocampal slices. Carbachol at concentrations of 10 μM or lower increased the frequency and decreased the amplitude of the discharges in a dose-dependent manner (Fig. 1A). The relative frequencies at 1, 5, and 10 μM carbachol were 2.82±0.15, 7.62±0.85, and 10.02±0.97, respectively. The relative amplitudes at 1, 5, and 10 μM carbachol were 0.85±0.07, 0.70±0.06, and 0.42±0.04, respectively. These alternations were statistically significant for 5 and 10 μM carbachol (n=5) (Fig. 1B). Carbachol at a concentration of 10 μM induced the bursts of the oscillation in the theta frequency range (11.20±1.10 Hz; n=3). After the theta bursts, the discharges were suppressed for a while.

Higher concentrations of carbachol induced intermittent episodes of prolonged field potential oscillations different from the epileptiform discharges. At 30 μM carbachol, the epileptiform discharge pattern was completely replaced by intermittent bursts of beta oscillations (Fig. 1A). The replacement of the epileptiform discharges with beta oscillations was first observed in the present study. The mean interburst interval of the oscillation was 19.73±3.32 sec (n=5). The mean frequency, amplitude, and duration of the oscillation were 15.50±0.75 Hz, 1.38±0.25 mV, and 9.91±0.77 sec, respectively (n=5). Thus, the frequency of the oscillation was within the beta oscillation range [9]. The amplitude of beta oscillations was significantly smaller than the amplitude of the epileptiform discharges recorded before the application of carbachol (paired t-test; ***p<0.001; n=5). The GABA\textsubscript{b} receptor antagonist, saclofen, did not block the induction of beta oscillations (data not shown; n=2).

Reduction in the evoked pEPSP slopes in the CA3 region by the cholinergic agonist

To investigate the effects of carbachol on excitatory synaptic connections, we measured pEPSPs evoked by stimulation of the Schaffer collaterals. In the presence of picrotoxin, the slope of the evoked pEPSPs decreased in a dose-dependent manner with the application of carbachol. The pEPSP slope recovered after the carbachol was washed out (Fig. 2A). The relative pEPSP slopes at 1, 5, 10, and 30 μM carbachol were 0.60±0.03, 0.41±0.02, 0.29±0.01, and 0.14±0.01, respectively. The reduction in the slopes of the evoked pEPSPs was significant at all the tested concentrations. After the carbachol was washed out, the relative pEPSP slope recovered to a value of 0.68±0.02 (n=6) (Fig. 2B).

The muscarinic cholinergic receptor is responsible for these effects

Carbachol is a broad-spectrum cholinergic agonist which acts at both nicotinic and muscarinic cholinergic receptors. We therefore investigated which of the cholinergic receptors contributes to changes in the epileptiform discharges and reduction in the evoked pEPSP slopes using selective cho-
linergic antagonists. A broad-spectrum muscarinic receptor antagonist, atropine sulfate (30 μM), was applied. In the presence of atropine sulfate, carbachol had no significant effect on the epileptiform discharges as shown in Figure 3. Both the frequency and amplitude of the epileptiform discharges moderately increased dependent on the concentration of carbachol; however, the increase was not significant at any concentration of carbachol. Carbachol at 10 μM did not induce theta oscillation. Higher concentrations of carbachol did not induce beta oscillations in the presence of atropine (n=6) (Fig. 3A). Carbachol had no effect on the evoked pEPSP slope with co-application of atropine (n=6) (Fig. 3B).

However, in the presence of the nicotinic receptor antagonist, d-tubocurarine (50 μM), carbachol increased the frequency of the epileptiform discharges, and decreased their amplitude at concentrations of 1, 5, and 10 μM in a dose-dependent manner (Fig. 4Aa). The relative frequencies at 1, 5, and 10 μM carbachol were 2.13±0.05, 4.20±0.23, and 8.54±0.69, respectively. The relative amplitudes at 1, 5, and 10 μM carbachol were 0.96±0.05, 0.85±0.06, and 0.50±0.03, respectively. The results for all the tested concentrations for the increase in frequency, and those for 5 and 10 μM for the decrease in amplitude (n=7) were statistically significant (Fig. 4Ab). Carbachol at 10 μM induced theta oscillation, and the higher concentration of carbachol induced beta oscillation. In the presence of d-tubocurarine, carbachol also decreased the evoked pEPSP slope in a dose-dependent manner. The relative pEPSP slopes at concentrations of 1, 5, 10, and 30 μM carbachol were 0.96±0.05, 0.57±0.04, 0.29±0.04, and 0.32±0.05, respectively. After the carbachol was washed out, the relative pEPSP slope recovered to 0.82±0.05 (n=4) (Fig. 4B) [31].
the cholinergic receptor activation decreased the number of hippocampal pyramidal neurons recruited into the synchronous firing in the epileptiform discharges. In addition, the application of carbachol depolarizes the membrane potential of the hippocampal pyramidal neurons [6] through the modulation of several ion channels such as the muscarinic-sensitive K⁺ channels and Ca²⁺-activated K⁺ channels [36,37]. The pyramidal neurons are facilitated to have spontaneous firings [38]. In results, each neuron in the population would have the uncorrelated firings. The uncorrelated neuronal firings in the population of neurons result in the smaller amplitude of the local field potentials compared to the correlated synchronous firings [32,33,39]. The amplitudes of theta and beta oscillations were smaller than that of the epileptiform discharges (Fig. 1A). In the generation of theta and beta oscillations, a relatively smaller number of pyramidal neurons could show correlated firing than that of them in the generation of the epileptiform discharges. In total, the activation of muscarinic ACh receptor would lead to the uncorrelated firings in the hippocampal pyramidal neurons, and it would decrease the amplitude of the discharges.

The strengthening of excitatory synaptic connections between pyramidal neurons is characteristic of the disinhibition model of epilepsy [29,40]. The CA3 region contains abundant excitatory recurrent connections [41], which are thought to be important for neuronal synchronization [42]. Hippocampal CA3 pyramidal cells tend to have the correlated firings using the recurrent excitatory synapses during the generation of the epileptiform discharges [43]. As described previously, carbachol would induce the uncorrelated firings of the CA3 pyramidal cells in the present study. Carbachol decreased pEPSP slope through muscarinic ACh receptors (Figs. 2, 3 and 4). It will inhibit EPSP between CA3 pyramidal cells. Even if EPSP is small, the activation of ACh receptors facilitates the spontaneous firings of the hippocampal pyramidal neurons [38]. In results, the application of carbachol induces the facilitation of the spontaneous firings of pyramidal neurons [38]. Perhaps each pyramidal neuron will separately have firings and has higher frequency of the firings after the application of carbachol. The epileptiform discharges have the threshold behavior [44]. The numbers of the firings of the pyramidal cells immediately reach the threshold and induces the epileptiform discharges. Hence, the frequency of the epileptiform discharges may increase with the application of carbachol. The computational study [45] found that the decrease in excitatory synaptic efficacy contributes to the modulation of epileptiform discharges.

Lower concentrations of carbachol facilitated the induction of epileptiform discharges, although the amplitude of the discharges became smaller. The increased frequency of the discharges is often thought to be associated with the facilitation of seizures [46,47]. When carbachol was applied at a higher concentration, the epileptiform discharges were totally suppressed and bursts of theta and beta oscillations
were induced instead of the discharges. In the previous study, the activation of the ACh receptor increases [31,48] and decreases [49,50] the frequency of the epileptiform discharges. The facilitation and suppression could be dependent on the muscarinic ACh receptor activation level.

One possible criticism is that the carbachol-induced beta oscillations resemble the ictal discharges of the epileptiform discharges, because the beta oscillations occurred like a burst. However, the ictal discharges induced by another cholinergic agent pilocarpine are reported to have frequencies between 4 and 10 Hz [51], whereas the carbachol-induced oscillations in the present study had relatively higher fre-

Figure 3  Atropine suppressed the effects of carbachol on epileptiform discharges and pEPSPs. Aa) Carbachol did not affect the epileptiform discharges in the presence of the muscarinic antagonist, atropine. Scale bars indicate 20 sec and 5 mV. Ab) The effect of carbachol on the relative frequency and amplitude of picrotoxin-induced epileptiform discharges in the presence of atropine. Ba) The effect of carbachol on the pEPSP slope and the relative pEPSP slope. The square area of the pEPSP in the top trace of Figure 2 is shown for each concentration. Scale bars indicate 2 ms and 0.5 mV; n=6 in both A and B.
In the previous study, Arai and Natsume (2007) reported that the co-application of 30 μM carbachol and 5 μM bicuculine (GABA_α receptor antagonist) not only decreases the frequency of beta oscillations in a burst, but also induces the significant differences between an ictal discharge and a burst of beta oscillations.

Furthermore, the CA3 region is recognized as the site that has a lesser incidence of ictal discharges in hippocampus [52]. The discharges are induced more frequently in the CA1 region [53]. In contrast, the occurrence of beta oscillations was robust in the CA3 region [9]. Our observations highlight the frequency of 13-20 Hz, that is, in the beta-frequency range. Figure 4 D-tubocurarine did not alter the effects of carbachol on epileptiform discharges and pEPSPs. A) d-tubocurarine did not alter the effects of carbachol on epileptiform discharges. Beta oscillations were induced at 1 and 10 μM carbachol. A) The effect of carbachol on the relative frequency and amplitude of the picrotoxin-induced epileptiform discharges in the presence of d-tubocurarine (*p<0.05, ***p<0.01, ANOVA and a post-hoc Fischer’s test; n=7). Scale bars indicate 20 sec and 2 mV. B) The effect of carbachol on the pEPSP slope (a) and the relative pEPSP slope (b). The pEPSPs from the onset of stimulation to 5 ms after are shown at each concentration. ***p<0.01; ANOVA and post-hoc Scheffe test; n=4. Scale bars indicate 2 ms and 0.5 mV.

In the previous study, Arai and Natsume (2007) reported that the co-application of 30 μM carbachol and 5 μM bicuculine (GABA_α receptor antagonist) not only decreases the frequency of beta oscillations in a burst, but also induces
theta oscillations. They concluded that the activation of the GABA<sub>A</sub> receptor is required for the generation of beta oscillations, and contributes to the regulation of the frequency of beta oscillations [9]. Recently, it is found that bicuculline plays the other role as an SK channel blocker than the GABA<sub>A</sub> receptor blocker [54]. After carbachol was added to the picrotoxin-induced epileptiform discharges, the beta oscillations were induced in the present study, and they were similar to those with the application of only carbachol (Fig. 1) [9]. Thus, the suppression of the GABA<sub>A</sub> receptor could not modulate the frequency of beta oscillation, but SK channel could modulate it. It may be necessary to confirm these results in further studies.

At the higher concentration, carbachol induced theta and beta oscillations. Then the epileptiform discharges were suppressed. The suppression mechanism of the epileptiform discharges by theta and beta oscillation has not yet been clarified. The oscillations occurred intermittently like a burst (Fig. 1). With the application of carbachol at 10 μM the discharges were suppressed for a while after the burst of theta oscillation (Fig. 1). Thirty μM carbachol induced the intermittent bursts of beta oscillations. We have thought that the burst of beta oscillations would have the suppression period for the epileptiform discharges after the generation of the burst of beta oscillation, and the burst would suppress the epileptiform discharges after the bursts. Further studies are necessary.

The induction of beta oscillations by 30 μM carbachol shows that muscarinic receptor activation can replace the epileptiform discharges in hippocampal slices with beta oscillations. The previous report found that the epileptiform discharges was suppressed by the generation of theta rhythms in the hippocampus in vivo [23,55]. These studies concluded that the “theta rhythm functional states” contribute to the antiepileptic effect [23,55]. In the present study, since beta oscillations were induced, the epileptiform discharges were suppressed (Fig. 1). Hence, the functional states with beta rhythms may also have an antiepileptic effect, similar to that observed with theta rhythms. Epileptic seizures preferentially occur during slow-wave phases of sleep, and are less frequent during wakefulness and rapid eye movement (REM) sleep [23,24]. The ACh levels in the hippocampus are high during wakefulness and REM sleep, and drop to a minimum during slow-wave sleep [25,26]. With the increase in ACh, EPSP between hippocampal pyramidal cells would be suppressed, the cells have more uncorrelated firings, theta or beta rhythm would be induced, and in results, epileptic seizures could be suppressed.

Conclusion

It was found that the activation of the cholinergic receptor altered the generation patterns of the epileptiform discharges, and that these effects were dependent on the activation level of the muscarinic receptors in hippocampus. With the lower activation levels of the receptors, more pyramidal cells would facilitated the firings, and in results they easily have the spontaneous firings. In results, the epileptic discharges would have the increased frequency, and smaller amplitude. With the higher activation levels, the discharges were replaced with the intermittent bursts of theta or beta oscillation with smaller amplitude. Dependent on the activation levels of ACh receptors, the epileptiform discharges in which the large number of the pyramidal cells have the correlated firing would be suppressed and the uncorrelated firings of them can be induced. Based on the idea of cholinergic control of epilepsy, new therapeutic approaches such as the stimulation of the medial septum or brain stem, and cell therapy, have been studied for temporal lobe epilepsy in the recent years [56–58].

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Conflicts of Interest

All authors declare that they have no conflict of interest.

Author Contributions

A. H. directed the research. T. S. and K. N. co-wrote the manuscript. T. S. and K. N. helped to draft the manuscript. All authors critically reviewed and approved the final manuscript.

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