CARBENOXOLONE DEPRESSION OF SPONTANEOUS EPILEPTIFORM ACTIVITY IN THE CA1 REGION OF RAT HIPPOCAMPAL SLICES

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Abstract—An important contributor to the generation of epileptiform activity is the synchronization of burst firing in a group of neurons. The aim of this study was to investigate whether gap junctions are involved in this synchrony using an in vitro model of epileptiform activity. Hippocampal slices (400 μm) were prepared from female Sprague–Dawley rats (120–170 g). The perfusion of slices with a medium containing no added magnesium and 4-aminopyridine (50 μM) resulted in the generation of spontaneous bursts of population spikes of a fast frequency along with less frequent negative-going bursts. The frequency of the bursts produced was consistent over a 3 h period. Carbenoxolone (100 μM), a gap junction blocker and mineralocorticoid agonist, perfused for 75 min, reduced the frequency of both types of spontaneous burst activity. Perfusion of spironolactone (1 μM), a mineralocorticoid steroid antagonist, for 15 min prior to and during carbenoxolone perfusion did not alter the ability of carbenoxolone to depress the frequency of spontaneous activity. The incubation of hippocampal slices in carbenoxolone prior to recording increased the time taken for the spontaneous activity to start on change to the zero magnesium/4-aminopyridine medium and decreased the total number of spontaneous bursts over the first 60 min period.

The ability of carbenoxolone to delay induction of epileptiform activity and reduce established epileptiform activity suggests that gap junctions contribute to the synchronization of neuronal firing in this model. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: hippocampus, connexin, gap junction.

Several theories have been proposed to explain the propensity of the hippocampus to seizure generation, including a reduction in inhibitory synaptic transmission, induction of recurrent synaptic excitation and non-synaptic mechanisms involving electrical transmission. Electrical effects may involve either ephaptic interactions (field effects) or specialized structures called gap junctions. Two mirror image hemichannels or connexons located in the plasma membrane of each cell form the gap junction. Hemichannels contain six homologous protein subunits called connexins (Cxs), of which more than a dozen have so far been cloned, with at least half expressed in the CNS. These gap junctions act as low pass filters and allow the passage of ions (Ca2+, K+), small molecules and second messengers (e.g., inositol, 1,4,5-triphosphate) between adjacent cells. Varying combinations of connexins produce gap junctions with diverse properties. Dye-coupling between cells using Lucifer Yellow and paired intracellular recordings have revealed the existence of gap junctions between pyramidal cells in the hippocampus. In the zero calcium model of epileptiform activity, procedures which block gap junctions also depressed seizure-like activity, suggesting that gap junctions play an important role in synchronizing neuronal firing in an in vitro model where Ca2+-dependent synaptic transmission is presumably blocked. The aim of this study was therefore to investigate whether gap junctions are involved in the induction and maintenance of epileptiform activity generated by a model in which chemical synaptic transmission is dominant.

EXPERIMENTAL PROCEDURES

For extracellular field recordings, female Sprague–Dawley rats (120–170 g) under halothane-induced anaesthesia were given a blow to the thorax and decapitated. The brain was removed into ice-cold artificial cerebrospinal fluid (ACSF). The hippocampi were dissected out and transverse slices (400 μm) were cut using a McIlwain tissue chopper. Hippocampal slices were kept in a storage chamber for at least 1 h before use. After such time, slices were transferred to an interface-type recording chamber constantly perfused (1.5 ml/min) with ACSF at 28–30°C. The normal ACSF contained in (mM): NaCl 124, KCl 3, NaHCO3 26, NaH2PO4 1.25, d-glucose 10, MgSO4 1 and CaCl2 2, saturated with 95% O2/5% CO2.

A bipolar stimulating electrode was placed in the stratum radiatum of the CA1 region to allow orthodromic stimulation of the Schaffer collateral commissural fibres. Field excitatory postsynaptic potentials (fEPSPs) were generated from the CA1 dendritic layer using borosilicate glass capillary electrodes (resistance 2–9 MΩ) filled with 3 M NaCl. An evoked fEPSP was obtained to check the viability of the slice and correct electrode positioning before the perfusion medium was changed to one containing no added magnesium and 4-aminopyridine (50 μM; 0Mg2+/4-AP) and no further stimulation was undertaken. In general, drugs were applied by addition to the perfusion medium. For intracellular acidification NaCl (30 mM) was substituted with sodium acetate (30 mM), and for intracellular alkalosis NaCl (10 mM) was replaced with ammonium chloride (10 mM). For the incubation studies, slices were incubated in the storage chamber in normal ACSF containing carbenoxolone (100 μM) for a period of at least 60 min prior to use. Control slices were treated in a corresponding manner without carbenoxolone. After the incubation period, slices were moved to the interface chamber and perfused with normal ACSF for a period of 15 min before the medium was changed to 0Mg2+/4-AP. The time taken from when the perfusing medium was changed to 0Mg2+/4-AP to the first burst of spontaneous activity and the total number of bursts in the first 60 min of 0Mg2+/4-AP perfusion were measured. Evoked fEPSP amplitude and spontaneous bursts of activity on a fast time scale were recorded using the LTP data program.
Materials

Carbenoxolone and octanol were obtained from Sigma. Spiranolac-tone was from RBI. Ammonium chloride and sodium acetate were from BDH. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), N-(2-amino-5-phosphono-pentanoic acid (AP5) and bicuculline were from Tocris.

RESULTS

Unless stated otherwise, all of the results relate to the extracellular field potential recordings. Perfusion of hippocampal slices with 0Mg²⁺/4-AP medium resulted in the generation of two types of spontaneous activity. These were characterized by either bursts of population spikes with an interburst frequency in the range of 0.5–1.5 Hz (referred to hereafter as interictal activity), or by large negative-going deflections with a much lower interburst frequency of 0.06–0.4 Hz (referred to hereafter as negative activity) (Fig. 1a, b). Under normal incubation conditions, spontaneous activity began 8.4 ± 0.7 min after change to 0Mg²⁺/4-AP medium (n = 6; Table 1). Once the spontaneous activity had started, a steady burst rate was soon established which varied little over a period of several hours (Fig. 2a). The N-methyl-D-aspartate (NMDA) receptor antagonist AP5 (40 μM), perfused for 15 min, had little effect on the rate of either interictal or negative bursts (rates were 83 ± 9% and 112 ± 16% of control, respectively, n = 3, both non-significant; Fig. 2b). CNQX (20 μM), a non-NMDA receptor antagonist, also failed to significantly affect the rate of negative burst activity, but reduced the frequency of interictal activity to 30 ± 3% of control (n = 3, P < 0.05; Fig. 2b). The GABA_A receptor antagonist bicuculline was used to further characterize the two types of spontaneous activity. Perfusion of bicuculline (100 μM) for 15 min increased the rate of interictal burst activity to 160 ± 13% of control (n = 3, P < 0.05), but totally inhibited negative burst activity in two of the three slices (17 ± 16% of control, n = 3, P < 0.05; Fig. 2c). Whole-cell recordings from six slices perfused with 0Mg²⁺/4-AP medium revealed only positive-going bursts, which consisted of multiple action potentials superimposed upon a sustained depolarization (Fig. 1c, d). These bursts started within 5–15 min of changing the medium and, like the extracellularly
recorded interictal activity, were blocked by perfusion of CNQX (20 μM, n = 5).

Gap junction block using carbenoxolone

Carbenoxolone, a gap junction blocker, was used to investigate the involvement of gap junctions in the burst activity caused by the 0Mg²⁺/4-AP conditions. In line with previous work, a concentration of 100 μM carbenoxolone was used in this study. Table 1 shows that, in slices incubated in carbenoxolone (100 μM) prior to being perfused with 0Mg²⁺/4-AP, the lag period between the start of 0Mg²⁺/4-AP perfusion and initiation of spontaneous activity was increased to 17.7 ± 0.8 min (n = 6). An unpaired Student’s t-test showed this to be a significant increase compared with slices incubated in normal ACSF (P < 0.0001). The total number of interictal bursts in the first 60 min of 0Mg²⁺/4-AP perfusion was also reduced in carbenoxolone incubated slices (55 ± 48, n = 6) compared with normal conditions (1179 ± 265, n = 6) (Table 1). Negative bursts were very infrequent in carbenoxolone-incubated slices.

The effect of carbenoxolone on established epileptiform activity was also investigated using slices in which spontaneous activity had already been induced by perfusion of 0Mg²⁺/4-AP medium. The action of carbenoxolone as a gap junction blocker is known to have a slow onset, with a lag time of 40 min being reported previously. Therefore, a long perfusion time of carbenoxolone was used to ensure that adequate time was allowed for gap junction blockade to ensue. When carbenoxolone (100 μM) was perfused for 75 min (Fig. 3a), interictal burst rate became significantly depressed after 40 min (n = 5, P < 0.05). Very little recovery occurred during washout of carbenoxolone and, after 120 min, the rate was 16 ± 7% of control (P < 0.01, n = 5).

Although carbenoxolone also decreased the rate of negative activity, it was only significant at one time-point using a

![Fig. 2. Characterization of interictal and negative burst activity. Part (a) monitors the rate of interictal and negative burst activity during a 180 min period from the start of perfusion of 0Mg²⁺/4-AP medium, which occurred at time zero. AP5 (40 μM) and CNQX (20 μM) were perfused for 15 and 10 min, respectively, with a 30 min wash period between subsequent perfusions (b). AP5 had little effect on either burst type, whereas CNQX significantly depressed the rate of interictal activity (P < 0.05, n = 3). Bicuculline (10 μM), perfused for 15 min, increased the rate of interictal activity whilst depressing that of negative-going activity (both P < 0.05, n = 3).](image-url)
Dunnett post hoc test (20 ± 20% of control 20 min after the end of carbenoxolone perfusion, $P < 0.05, n = 3$). Intercitial activity generated by perfusion of either component of our modified medium alone, i.e. zero magnesium or 4-AP (50 $\mu$M), was also depressed extensively by carbenoxolone (data not shown). In separate experiments, the amplitude of fEPSPs recorded from the CA1 region under low-frequency stimulation conditions and perfusion of normal ACSF was not altered by perfusion with carbenoxolone (100 $\mu$M; data not shown).

Mineralocorticoid antagonism using spironolactone

In order to verify that the action of carbenoxolone was due to its acting as a gap junction blocker and not as a mineralocorticoid agonist, spironolactone, a mineralocorticosteroid antagonist, was used. Spironolactone (1 $\mu$M) was perfused for 15 min prior to, and during, the 75 min of carbenoxolone (100 $\mu$M) perfusion. In the presence of spironolactone, carbenoxolone still significantly depressed the rate of spontaneous interictal activity ($P < 0.05, n = 4$; Fig. 3c). Again, washing for 120 min did not result in any noticeable recovery (burst rate was 11 ± 5% of control at 120 min after the end of drug perfusion, $P < 0.01, n = 4$). As with carbenoxolone alone, the perfusion of carbenoxolone together with spironolactone had a smaller effect on negative burst activity, which only became significant 20 min after the end of drug perfusion. Perfusion of spironolactone (1 $\mu$M) alone for 90 min tended to increase the rate of negative burst firing, but this was short lasting and statistically insignificant, and the rate of the interictal bursts remained constant (Fig. 3b).

Gap junction block using intracellular pH changes and octanol

Three other established means of blocking gap junctions were investigated. Intracellular acidification was achieved by perfusing a medium in which 30 mM NaCl was replaced with...
30 mM sodium acetate for 15 min. Burst rate was not significantly changed during or after perfusion with this medium (Fig. 4a). In one of the four slices, a large increase in negative-going burst activity occurred after sodium acetate perfusion. The replacement of NaCl (10 mM) with an equivalent molarity of ammonium chloride causes an intracellular alkalosis with a resulting acidification on washout of the ammonium chloride-containing medium. Interictal burst activity was almost totally inhibited after 10 min perfusion of test medium (5 ± 5% of control). However, this inhibition did not display the same gradual decline in burst rate that was characteristic with carbeneoxolone, but instead was very sudden and was accompanied by an increased level of noise on the trace (Fig. 4b). In one of the four slices, an increase from 36 to 98 bursts of interictal activity per minute occurred during perfusion of ammonium chloride and prior to total inhibition. Negative burst activity was also reduced, but far less dramatically (reduced to 63 ± 10% of control, n = 4, P < 0.05). In two of four slices this level of depression was maintained throughout the 30 min wash period. The third gap junction blocker used was octanol. Slices were perfused sequentially with octanol (0.14 and 0.7 mM) for 15 min per concentration (Fig. 5a). Octanol (0.14 and 0.7 mM) reduced interictal burst rate to 64 ± 7% and 45 ± 6% of control (P < 0.01, n = 4), respectively. Octanol had the opposite effect on negative activity, increasing burst rate to 123 ± 8% and 197 ± 40% of control (P < 0.05, n = 4) with respect to increasing concentration. Only a very small degree of recovery occurred during the 60 min wash with 0Mg2+/4-AP. The solvent for octanol was ethanol. Slices were therefore exposed to 0.02% and 0.1% ethanol, corresponding to 0.14 and 0.7 mM octanol. Perfusion with ethanol at these concentrations for 15 min in succession had no significant effect on burst rate (Fig. 5b).

**DISCUSSION**

*In vitro* models of epileptiform activity predominantly involve the generation of interictal-like activity that is so called owing to the resemblance to abnormal wave activity seen in an electroencephalogram between seizures. The intracellular correlate is a paroxysmal depolarizing shift that consists of depolarization of the cellular membrane along with bursts of action potentials. A combination of zero magnesium and 4-AP was used to generate epileptiform activity of an interictal-like nature in this study. Similar activity was generated using this model in the CA3 region of rat hippocampal and neocortical slices. The whole-cell recordings showed these bursts to be composed of action potentials superimposed on a depolarizing shift similar to those induced by a combination of zero magnesium and 4-AP in the CA3 subfield of the hippocampus and in neocortical neurons. In addition to the regularly occurring interictal-like bursts of activity, less frequent large negative bursts that were inhibited by bicuculline also appeared in the majority of our slices. Similar large negative-going potentials associated with a rise in extracellular potassium were generated in addition to interictal activity in the CA3 region of hippocampal slices perfused with 4-AP (50 μM). As in this study, these potentials were blocked by bicuculline, suggesting that these are GABA-mediated potentials. No such negative bursts were observed using the whole-cell recordings. This may have been a result of the lower temperature at which these slices were maintained, or the age difference in the animals from which they were prepared, but whatever the reason, this prevented us characterizing the negative bursts further.

The combination of zero magnesium and 4-AP generates a model of epileptiform activity that involves multiple
mechanisms responsible for its induction and maintenance. Zero magnesium conditions raise neuron excitation mainly by removing the voltage-dependent block of NMDA receptors which is present under normal conditions, and hence the inhibition of activity by NMDA receptor antagonists, although a reduction in membrane charge screening and a diminished antagonism between magnesium and calcium also play a role. 4-AP at the concentration used in this study inhibits the D potassium current, thus prolonging membrane depolarization and hence calcium influx and transmitter release. Infusion of 4-AP into the hippocampus of rats in vivo produced a substantial increase in glutamate release. A direct 4-AP-mediated effect on voltage-gated calcium channels has also been proposed.

In our hands, CNQX, a non-NMDA receptor antagonist, depressed interictal activity significantly whilst not affecting the negative bursts, which is in agreement with the study of Avoli et al. Under the conditions in this study, the NMDA receptor antagonist AP5 only reduced the frequency of interictal activity slightly and in our small sample this did not reach statistical significance. However, it should be noted that, while perfusion of CNQX alone substantially blocked interictal activity, perfusion of both CNQX and AP5 was required to block it totally, suggesting that both NMDA and non-NMDA receptors are required for this activity.

The incidence of dye-coupling between CA1 pyramidal neurons was increased in both a calcium-free and a tetanus toxin model of epileptiform activity. Elevated levels of Cx43 and to a lesser extent Cx32 were found in samples of temporal lobe neocortex from patients undergoing surgical resection for intractable seizure disorder. Both Cx43 and Cx32 are expressed in the adult rat hippocampus. However, in contrast, Eliseevich et al. found no up-regulation of Cx43 in patients suffering from frontal lobe epilepsy, suggesting that a change in dynamic channel properties rather than number may be involved. Although the interictal-like activity generated by 0Mg2+/4-AP is highly dependent on the activation of non-NMDA receptors, it is possible that gap junctions may also be involved. Glycyrrhetinic acid, an aglycone sapo-nin extracted from liquorice root, is a potent gap junction blocker, and several derivatives have been synthesized which display varying degrees of ability to inhibit gap junctions. Carbenoxolone, 18β-glycyrrhetinic acid 3β-O-hemisuccinate, is reported to block gap junction communication in human fibroblasts with an IC50 of 3 μM, in rat sympathetic preganglionic neurons and in CNS precursor cells, NT2/D1. It has been proposed that glycyrrhetinic acid derivatives bind directly to the gap junction, inducing a conformational change and subsequent channel closure, and this is supported by freeze-fracture analysis using glioma cells and 18α-carbenoxolone.

Under control conditions, the perfusion of 0Mg2+/4-AP resulted in the generation of spontaneous bursts of activity within an average of 10 min, which is in agreement with previous studies. However, the time to burst induction was more than doubled in slices incubated in carbenoxolone, suggesting that gap junctions may be involved in generating the synchronization of increased cell excitability, which is necessary for burst activity. Perfusion of carbenoxolone also irreversibly decreased the frequency of established interictal activity after about 40 min perfusion, which is in accord with its time course of action on electrical coupling in sympathetic preganglionic neurons. Although some variation was apparent, in general the rate of the negative potentials was also depressed by carbenoxolone. In other studies, 18α-carbenoxolone (20 μM) reversibly abolished spontaneous interictal spikes induced by bicuculline in the anterior piriform cortex, and secondary but not primary discharges
generated by exposure to 0Mg2+ medium were abolished by carbenoxolone (100 μM) in the CA3 region of hippocampal slices.17

The lack of recovery in the present study, even after extensive washing, suggests that carbenoxolone could have had a deleterious effect on the hippocampal slices. In fibroblast cells, carbenoxolone had a Tc50 of 50 μM;4 however, in our experiments, as in the studies of Leslie et al.28 and Köhling et al.,17 a concentration of 100 μM was used. Arguing against our results being due to a general toxic action is the fact that a 75 min perfusion of carbenoxolone did not impair fEPSP amplitude recorded from hippocampal slices in standard ACSF.

In addition to being a gap junction blocker, carbenoxolone is also a mineralocorticoid agonist and mineralocorticoid receptors are found in the CA1 hippocampal region. Activation of such receptors by mineralocorticoid agonists or low concentrations of corticosterone is generally excitatory in nature, causing a decrease in spike frequency adaptation,15 and increasing susceptibility to convulsions induced by pentyleneetetrazol and kainate.32 This is therefore in complete contrast to the action of carbenoxolone noted in this study. However, in order to establish that the action of carbenoxolone was not through activation of mineralocorticoid receptors, spironolactone, a mineralocorticoid antagonist, was used. In the study by Joels and de Kloet,15 spironolactone (100 mM) inhibited the excitatory effects of corticosterone. Spironolactone neither inhibited the action of carbenoxolone on burst frequency nor produced any significant effect when perfused alone, firstly verifying that carbenoxolone in this instance is not acting via mineralocorticoid receptors and, secondly, suggesting that such receptors are not involved in the induction or maintenance of spontaneous activity generated by 0Mg2+/4-AP.

Dye-coupling and hence gap junction conductance is modulated by changes in intracellular pH, with acidification reducing and alkalization enhancing junctional conductance.6,29,40 In the present study, the perfusion of a medium in which 30 mM NaCl had been replaced with sodium acetate, which should result in intracellular acidification, had no effect on the frequency of either type of spontaneous activity induced by 0Mg2+/4-AP. In slices of cortex prepared from seven-day-old rats, sodium propionate (30 mM) caused an intracellular acidic pH shift of 0.37 ± 0.02 pH units.31 In crayfish muscle, acetate lowered internal pH by 0.57 pH units, which was marginally greater than the 0.5 pH unit acidification achieved by propionate.37 In a calcium-free model of bursting, propionate decreased the percentage of coupled cells and abolished all spontaneous activity.31 Not all Cx subunits are equally sensitive to pH modulation, such that Cx43 is more sensitive than Cx32;21 thus, it is possible that the gap junctions involved in governing burst activity are not sensitive to this type of modulation. In argument against this theory is the fact that acidification using propionate reduced dye-coupling in the hippocampal CA1 subfield.31

It was possible that, for some reason, the replacement of NaCl with sodium acetate did not result in the proposed acidification; therefore, a different way of approaching pH alteration was attempted. Ammonium chloride induces a transient intracellular alkalization followed by acidification upon washout,13,42 hence opening and closing gap junctions in succession. The frequency and duration of spontaneous field activity in a zero calcium model was increased during, and suppressed following, ammonium chloride (10 mM) perfusion.31 In the present study, a clear increase followed by complete suppression of interictal activity was seen in only one of the four slices exposed to ammonium chloride. In a separate report, an increase in the number of discharges accompanying spontaneous interictal spikes generated by bicuculline occurred within 2 min of ammonium chloride perfusion, followed by a silent period after 10 min perfusion.10 In the same study, ammonium chloride perfusion also caused a disruption in interictal spike synchronization, causing an increased number of smaller discharges. In our study, the decrease in interictal activity was accompanied by an increase in the baseline noise of the trace, which could be interpreted as resulting from an initial increase in burst number, but also a severe decrease in burst size, making each single burst indistinguishable from the next.

A further blocker of gap junctions is the n-alcohol, octanol.30 Under conditions of zero calcium, octanol (0.2 mM) stopped field burst activity without reducing the frequency of cell firing in hippocampus.31 In cortical slices, perfusion of octanol completely abolished spontaneous interictal activity.30 Octanol at a higher concentration of 1–2 mM blocked spreading depression propagation induced by microinjection of KCl in either a partial or fully reversible manner.19 The reduction in spontaneous interictal burst activity by octanol in our experiments is therefore in correlation with the effect of carbenoxolone. In complete opposition to the effect on interictal activity, octanol increased the rate of the GABAergic-mediated negative bursts in the present study. Octanol is non-specific, with an ability to generate currents that are inhibited by bicuculline and thus attributed to the activation of GABA<sub>A</sub> receptors.18 Octanol (0.02 mM) also decreased the mean open time for NMDA channels in hippocampal neurons whilst increasing the amplitude of unitary currents.24 Therefore, the possibility exists that the action of octanol may be through mechanisms other than block of gap junctions.

CONCLUSIONS

The perfusion of hippocampal slices with a medium containing no added magnesium and 4-AP (50 μM) generates spontaneous interictal activity and negative bursts in the CA1 dendritic subfield. Both the induction of burst activity and established epileptiform activity are subject to inhibition by the gap junction blocker carbenoxolone, thus suggesting that gap junctions contribute to the synchronization of neuronal firing that underlies seizure generation.

Acknowledgements—We thank the Wellcome Trust and the Epilepsy Research Foundation for support.

REFERENCES


(Accepted 12 July 2000)