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Pharmacological modulation of $GABA_A$ receptor-mediated postsynaptic potentials in the CA1 region of the rat hippocampus

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1 It is unclear whether $GABA_A$ receptor-mediated hyperpolarizing and depolarizing synaptic potentials ($IPSP_A$s and $DPSP_A$s, respectively) are evoked by (a) the same populations of GABAergic interneurones and (b) exhibit similar regulation by allosteric modulators of $GABA_A$ receptor function. We have attempted to address these questions by investigating the effects of (a) known agonists for presynaptic receptors on GABAergic terminals, and (b) a range of $GABA_A$ receptor ligands, on each response.

2 The GABA uptake inhibitor NNC 05-711 (10 μM) enhanced whereas bicuculline (10 μM) inhibited both $IPSP_A$s and $DPSP_A$s.

3 (−)-Baclofen (5 μM), [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO; 0.5 μM), and carbachol (10 μM) caused substantial depressions (up to 99%) of $DPSP_A$s that were reversed by CGP 55845A (1 μM), naloxone (10 μM) and atropine (5 μM), respectively. In contrast, 2-chloroadenosine (CADO; 10 μM) only slightly depressed $DPSP_A$s. Quantitatively, the effect of each agonist was similar to that reported for $IPSP_A$s.

4 The neurosteroid ORG 21465 (1–10 μM), the anaesthetic propofol (50–500 μM), the barbiturate pentobarbitone (100–300 μM) and zinc (50 μM) all enhanced $DPSP_A$s and $IPSP_A$s.

5 The benzodiazepine (BZ) agonist flunitrazepam (10–50 μM) and inverse agonist DMCM (1 μM) caused a respective enhancement and inhibition of both $IPSP_A$s and $DPSP_A$s. The $BZ\omega_1$ site agonist zolpidem (10–30 μM) produced similar effects to flunitrazepam.

6 The anticonvulsant loreclezole (1–100 μM) did not affect either response.

7 These data demonstrate that similar populations of inhibitory interneurones can generate both $IPSP_A$s and $DPSP_A$s by activating $GABA_A$ receptors that are subject to similar allosteric modulation.

Keywords: Baclofen; carbachol; DAGO; benzodiazepine; $GABA_A$ receptors; ORG 21465; propofol; zinc; zoplidem

Introduction

Synaptic activation of γ-aminobutyric acid $(GABA)_A$ receptors classically produces a fast hyperpolarizing inhibitory postsynaptic potential ($IPSP_A$) that is mediated by a chloride conductance. However, during periods of repetitive stimulation at frequencies in excess of 20 Hz (Davies & Collingridge, 1993; Grover *et al*., 1993; Staley *et al*., 1995; Taira *et al*., 1997) or in the presence of certain pharmacological agents e.g. pentobarbitone or 4-aminopyridine (Alger & Nicoll, 1982a; Avoli & Perreault, 1987; Perreault & Avoli, 1988; Michelson & Wong, 1991; Lambert *et al*., 1992; Xie & Smart, 1993; Kaila, 1993), an additional $GABA_A$ receptor-mediated depolarizing postsynaptic potential ($DPSP_A$) can be evoked. The $DPSP_A$ is much slower than the $IPSP_A$ and whilst the $DPSP_A$ substantially shunts other synaptic potentials, e.g. glutamate-mediated excitatory postsynaptic potentials (EPSPs), it can be sufficiently large to trigger the firing of multiple action potentials (Taira *et al*., 1997). In addition, this synaptic potential has been shown to be instrumental in initiating ictal discharges in an *in vitro* model of temporal lobe epilepsy (Avoli *et al*., 1996). As such, activation of $GABA_A$ receptors has both excitatory and inhibitory functions in the CA1 region of the hippocampus. Thus, selective modulation of one or other of these functions may have important implications clinically in terms of developing anxiolytic, anaesthetic and anticonvulsant drugs with greater effectiveness and reduced side effects.

Whilst the mechanism generating $IPSP_A$s is universally accepted to be a $GABA_A$ receptor-mediated increase in Cl^- conductance there has been extensive debate as to the mechanisms underlying activation of $DPSP_A$s. A number of studies have addressed the ionic mechanism underlying this response. However, to date, there is no clear consensus as to the precise mechanism(s) involved. As such, it has been proposed that the $DPSP_A$ arises from (1) an asymmetric, activity-dependent collapse of opposing chloride and bicarbonate electrochemical gradients across $GABA_A$ receptors (Staley *et al*., 1995), (2) an activity-induced extracellular K^+ transient that is generated in a bicarbonate-dependent manner by a local inhibitory network (Kaila *et al*., 1997) and (3) activation of different $GABA_A$ receptors, possibly activated by different types of interneurone (Perkins & Wong, 1996).

Since different interneurone populations are differentially susceptible to presynaptic regulation by a variety of neurotransmitters (Freund & Buzsáki, 1996) we have attempted to address whether $DPSP_A$s and $IPSP_A$s are differentially regulated presynaptically by evaluating the effects on the $DPSP_A$ of a range of agonists that have been reported to presynaptically depress the $IPSP_A$. In addition, we have tested the effects of a range of postsynaptic allosteric modulators of $GABA_A$ receptor function on the $DPSP_A$ and qualitatively compared these to their effects on the $IPSP_A$.

Preliminary reports of some of these findings have been published in abstract form (Manuel & Davies, 1997a,b).

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Methods

Biological preparation

Experiments were performed on hippocampal slices obtained from Wistar rats (3–4 weeks old) as described previously (Davies *et al*., 1990). In brief, animals were cervically dislocated and subsequently decapitated in accordance with U.K. Home Office guidelines. The brain was removed rapidly and transverse slices (400 μM thick) containing hippocampus were cut using a Campden vibroslicer. The hippocampal region was dissected from these slices and area CA3 removed. Two of the resultant CA3-ectomized hippocampal slices were immediately transferred to an interface recording chamber maintained at 30–32°C whilst the others were stored at room temperature for later use. Slices rested on a nylon mesh at the interface of a warmed perfusing artificial cerebrospinal fluid containing (mM): NaCl 124; KCl 3; $NaHCO_3$ 26; $CaCl_2$ 2; $MgSO_4$ 1; D-glucose 10; NaH_2PO_4 1.25, bubbled with a 95% O_2/5% CO_2 mixture.

Electrophysiological recording

Intracellular recordings were obtained from neurones in stratum pyramidale using glass microelectrodes (60–120 MΩ) filled with potassium methylsulphate (2 M) connected to an Axoclamp-2A amplifier used in discontinuous current-clamp mode (Axon Instruments, Foster City, CA, U.S.A.). Input resistances of pyramidal cells were routinely measured throughout each experiment by passing negative current pulses (amplitude 0.3–0.6 nA, duration 300 ms) through the intracellular recording electrode every 60–120 s. In all experiments 6-nitro-7-sulphamoylbenzo-[f]-quinoxaline-2,3-dione (NBQX; 3 μM), D-(E)-2-amino-4-methyl-5-phospho-3-pentanoic acid (CGP 40116; 50 μM) and ketamine (50 μM) were present in the perfusing medium to block all ionotropic glutamate receptor-mediated synaptic transmission. Monosynaptic $IPSP_A$s and $DPSP_A$s were evoked by delivering a single stimulus and 10 stimuli at 100 Hz, respectively, using bipolar stimulating electrodes placed in stratum oriens and stratum radiatum close to the recorded neurone. Stimulation strengths were set such that baseline synaptic responses had peak amplitudes approximately 50% of maximum. To quantify the effects of drugs, synaptic responses were compared before and after drug treatment at a fixed membrane potential. This was achieved by injecting DC to compensate for any drug-induced changes in membrane potential. Unless otherwise stated, the membrane potential at which $IPSP_A$s were studied was between −60 mV and −65 mV and at which $DPSP_A$s were investigated equivalent to the reversal potential of the $IPSP_A$ in each individual neurone, i.e. approximately −76 mV. This latter procedure ensured that an accurate measurement of the $DPSP_A$ amplitude could be obtained because an $IPSP_A$ was not superimposed on this response. Often the effects of drugs on $IPSP_A$s and $DPSP_A$s in the same cell were studied by switching between these two membrane potentials and between single shock and burst stimulation.

Drugs

Drugs were administered by addition to the superfusing medium and were applied for a sufficient period (15–20 min) to allow their full equilibration. Atropine, (−)-baclofen, bicuculline methiodide, 2-chloroadenosine (CADO), [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin (DAGO), flunitrazepam, picrotoxin, propofol, zinc and (N,N,6-trimethyl-2-(p-tolyl)-imidazo[1,2-a]pyridine-3-acetamide (zolpidem) were obtained from Sigma. Methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) was obtained from Research Biochemicals International. 6-nitro-7-sulphamoylbenzo-[f]-quinoxaline-2,3-dione (NBQX) was purchased from Tocris-Cookson. D-(E)-2-amino-4-methyl-5-phospho-3-pentanoic acid (CGP 40116) and [1-(*S*)-3,4-dichlorophenyl) ethyl] amino-2-(S)-hydroxypropyl-*p*-benzyl-phosphonic acid (CGP 55845A) were gifts from Dr M.F. Pozza and Dr W. Froestl at Novartis Pharma AG, Basle, Switzerland. Diphenylmethanone, 0-[2-(3-carboxy-1,2,5,6-tetrahydro-1-pyridinyl)ethyl]oxime hydrochloride (NNC 05-711) was a gift from Dr H. Mengel, Novo-Nordisk, Denmark. (2β, 3α, 5α)-3-hydroxy-2-(2,2-dimethylmorpholin-4-yl)pregnane-11,20-dione (ORG 21465) was a gift from Dr D.R. Hill, Organon Laboratories Ltd., Newhouse, Lanarkshire, U.K. Loreclezole was a gift from Dr M. Janssen, Janssen Pharmaceutica, Beerse, Belgium. Each drug was dissolved in distilled water, equimolar NaOH, or DMSO except loreclezole which was dissolved in 1 equivalent tartaric acid solution plus 10% v/v hydroxypropyl-β-cyclodextrin. Stock solutions of drugs were made up at 100–1000 times their final bath applied concentration. Where necessary, vehicle controls were performed to establish that drug effects related to the drug *per se* and not to the vehicle.

Analysis

Results were quantified in terms of drug-induced changes in peak amplitudes of $DPSP_A$s and $IPSP_A$s or decay time constants of $IPSP_A$s. As such, data are expressed as the magnitude of these parameters in the presence of the drug at equilibrium as a percentage of the corresponding control value where 100% equals no change. Decay time constants of $IPSP_A$s were assessed by fitting a first order exponential function to the decay of the $IPSP_A$ from its peak using the Chebyshev method in the Clampfit 6 suite of analysis programs (Axon Instruments). Statistical evaluation of raw data was made difficult by the variable size of the control responses from one neurone to another. As such, pooled data are presented as means ± standard error of the mean (s.e.mean). *n* signifies the number of times a result was obtained, which was the same as the number of slices tested. Each slice was obtained from a separate rat.

Results

The present data were collected from 98 neurones with resting membrane potentials more negative than −55 mV, overshooting action potentials, and resting input resistances in the range 30–60 MΩ.

Characterization of $DPSP_A$s and $IPSP_A$s

In the combined presence of the $GABA_B$ receptor antagonist CGP 55845A and the ionotropic glutamate receptor antagonists NBQX, CGP 40116 and ketamine, a single stimulus delivered in stratum oriens or stratum radiatum evoked a fast hyperpolarizing inhibitory postsynaptic potential (IPSP) in every cell recorded ($n=98$; Figure 1a). In each of these cells, ten stimuli delivered at 100 Hz, in either dendritic field, evoked a hyperpolarizing response that was followed by a slow depolarizing reponse at the resting membrane potential of the cell (not illustrated) and, a pure depolarizing response when the cell

was held at the reversal potential of the hyperpolarizing IPSP (Figure 1a). For ease of identification, the hyperpolarizing response will be referred to as an $IPSP_A$ and the depolarizing response as a $DPSP_A$. No difference in the waveform or ease of evoking a $DPSP_A$ or an $IPSP_A$ in either dendritic field was observed. However, unlike $IPSP_A$s

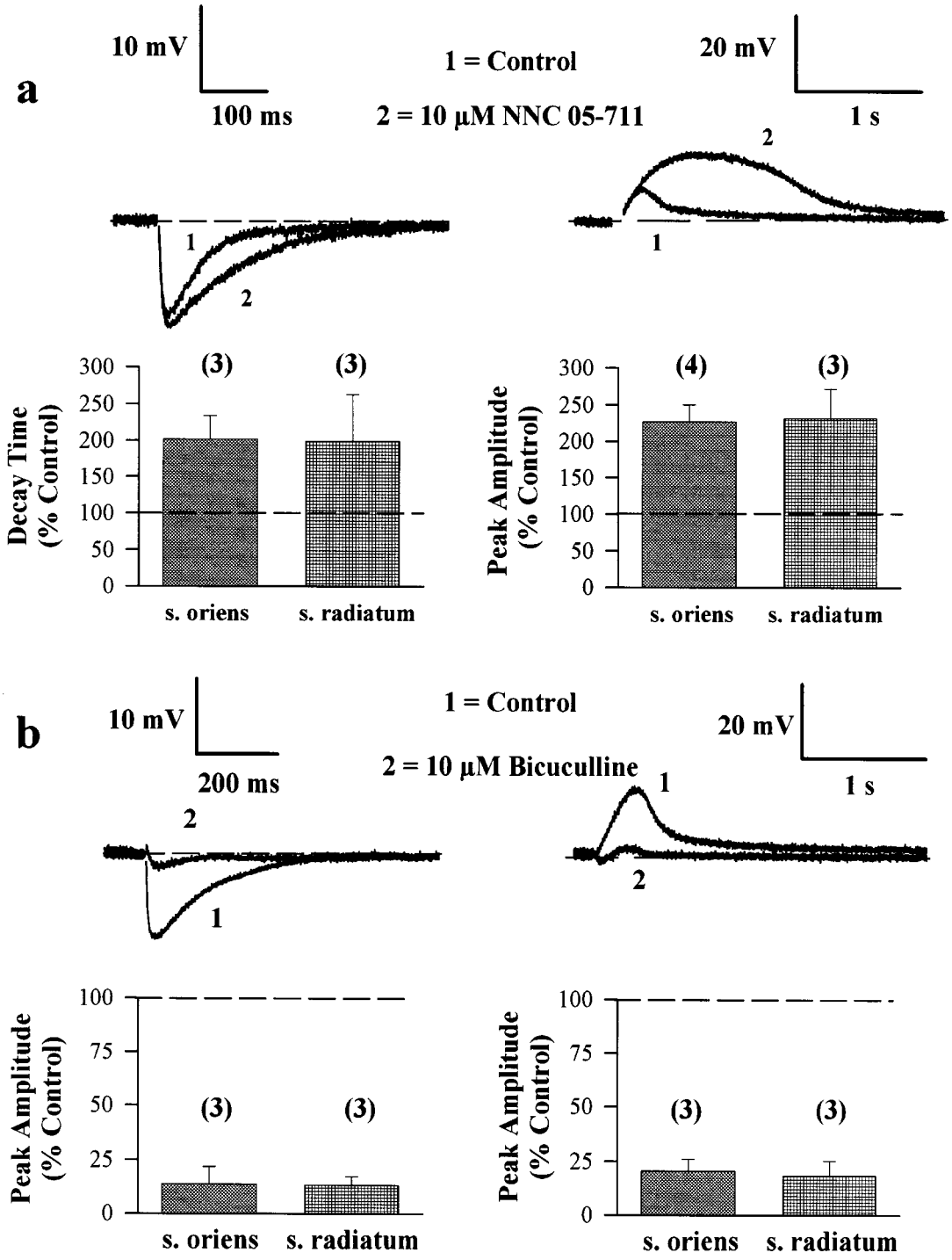

Figure 1 Identification of the $IPSP_A$ and the $DPSP_A$ as $GABA_A$ receptor-mediated responses. In (a) left-hand traces represent superimposed $IPSP_A$s evoked in the presence and absence of 10 μM NNC 05-711. Right-hand traces represent $DPSP_A$s under the same conditions obtained from a different neurone. In this and all subsequent figures synaptic traces in the presence and absence of a drug were recorded at the same membrane potential and stimulus artefacts are blanked for clarity. All synaptic traces are averages of 4–5 successive $IPSP_A$s or $DPSP_A$s. The bar graphs below the sets of synaptic traces illustrate pooled data for the effect of NNC 05-711 on the decay time constant of $IPSP_A$s and the peak amplitude of $DPSP_A$s evoked by stimulation in either stratum oriens or stratum radiatum. Each bar represents the averaged decay time constant of the $IPSP_A$ or peak amplitude of the $DPSP_A$ measured over a 10 min period at the peak of the drug effect expressed as a percentage of the corresponding parameters averaged over a 10 min period prior to drug application. Values are means $\pm$ s.e.mean such that 100% is equivalent to no change and the number of times each experiment has been performed is given in parentheses. This method of quantifying drug effects is used to generate all bar graphs illustrated in subsequent figures. Note that NNC 05-711 enhanced the duration and amplitude of the $DPSP_A$ and the decay time constant of the $IPSP_A$. (b) is a similar format to (a) and illustrates antagonism of $IPSP_A$s (left hand traces and bar graph) and $DPSP_A$s (right-hand traces and bar graph) by 10 μM bicuculline.

which could be activated reproducibly every 15 s, reproducible $DPSP_As$ could be evoked using burst stimulation only once every minute as faster rates of stimulation caused depression of responses. As such, if stimulation in stratum radiatum 30 s after stimulation in the stratum oriens caused no depression of synaptic responses the two inputs were deemed to be independent. Indeed, extracellular recording and current source density analysis has demonstrated that this method of stimulation ensures that the majority of GABA-mediated synaptic current is generated horizontal to

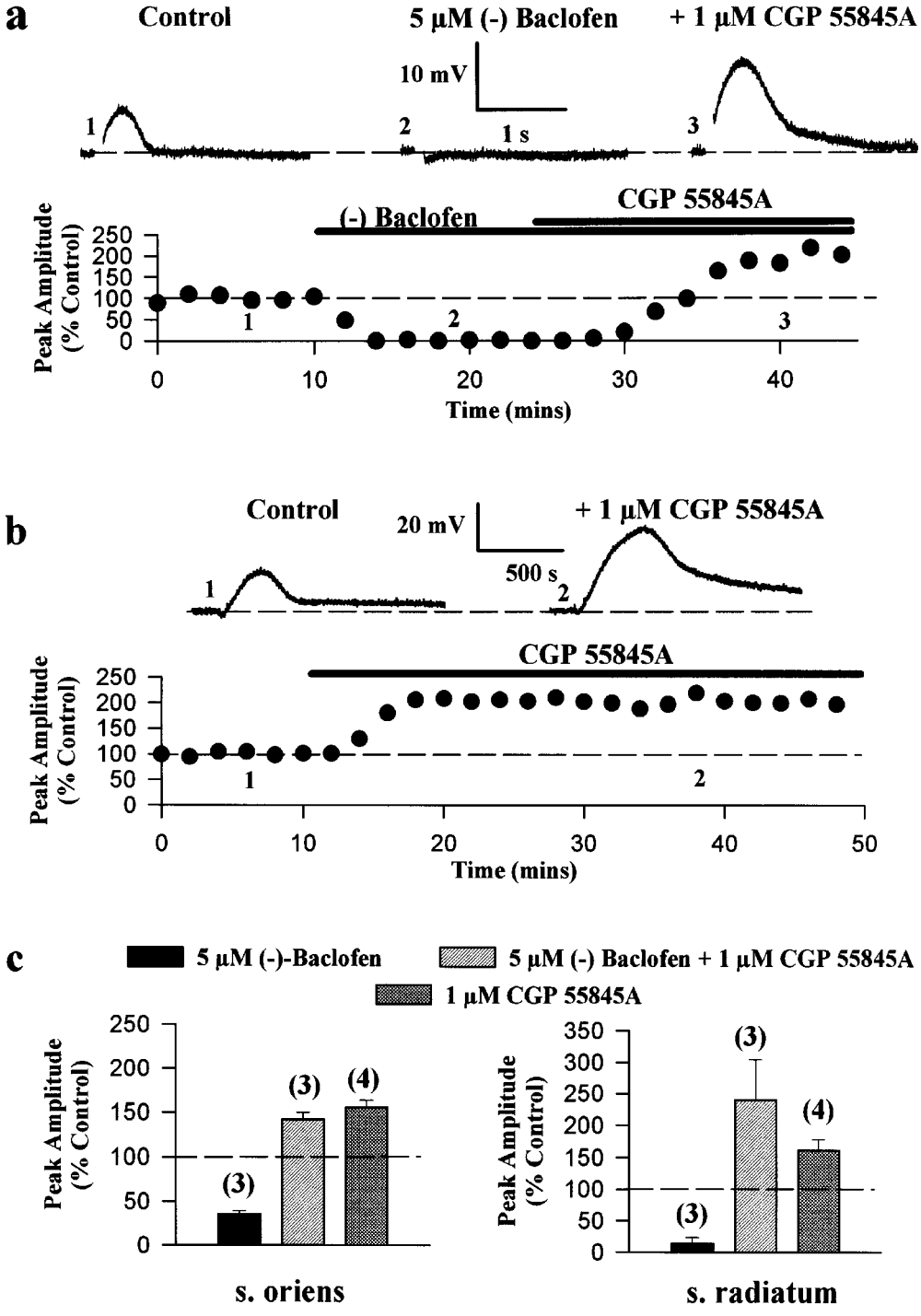

Figure 2 $GABA_B$ receptor modulation of $DPSP_As$. In (a) synaptic traces are $DPSP_As$, evoked in response to a burst of ten stimuli delivered at 100 Hz, recorded in control medium (1), in the presence of 5 μM (−)-baclofen (2) and following addition of 1 μM CGP 55845A (3). The graph shows a plot of the peak amplitudes of successive $DPSP_As$ normalized to the mean peak amplitude of the five $DPSP_As$ prior to (−)-baclofen application *versus* time for a single experiment. (−)-Baclofen and CGP 55845A were applied for the times indicated by the bars. 1, 2 and 3 refer to the synaptic traces illustrated above the graph. (b) shows data from another experiment in a different neurone in which the effect of 1 μM CGP 55845A alone was tested. Traces are $DPSP_As$ recorded in the absence (1) and presence (2) of CGP 55845A and the graph illustrates the time course of the facilitatory effect of CGP 55845A on the $DPSP_A$ in this cell. The bar graphs in (c) illustrate pooled data for the effect of 5 μM (−)-baclofen, 5 μM (−)-baclofen + 1 μM CGP 55845A and 1 μM CGP 55845A alone on $DPSP_As$ evoked by stimulation in stratum oriens (left-hand graph) and stratum radiatum (right-hand graph).

the stimulation site (Lambert *et al.*, 1991a), i.e. in basal dendrites in the case of stratum oriens stimulation and apical dendrites in the case of stratum radiatum stimulation.

To establish that both the $IPSP_A$ and the $DPSP_A$ were mediated by the release of GABA we tested the effects of the GABA uptake inhibitor NNC 05-711 on both responses in each dendritic field. NNC 05-711 (10 μM) prolonged the $IPSP_A$ without substantially affecting its amplitude and enhanced both the peak amplitude and the duration of the $DPSP_A$ ($n=3-4$ for both $IPSP_A$ and $DPSP_A$; Figure 1a). In contrast, bicuculline (10 μM) depressed reversibly both

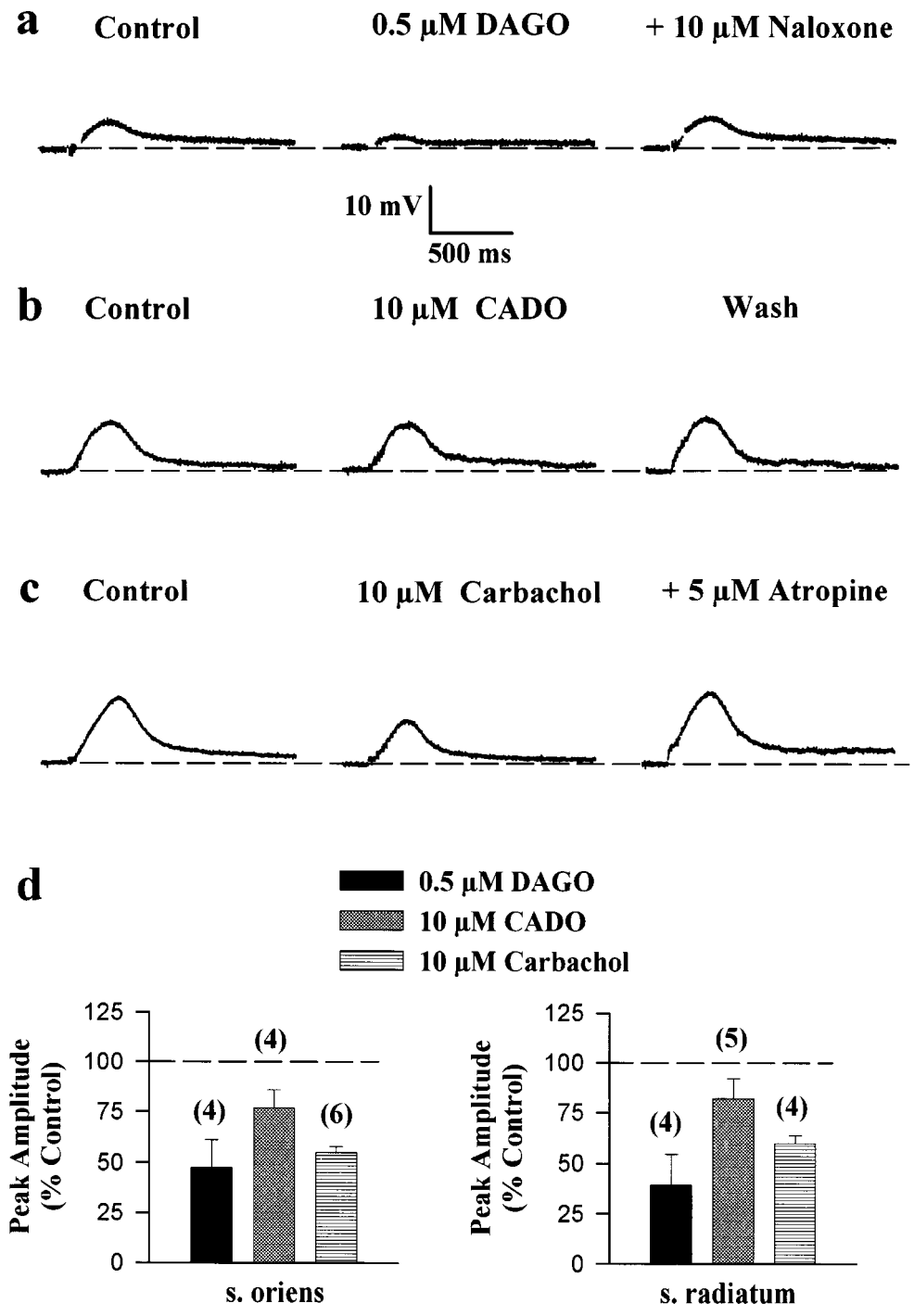

Figure 3 Effect of DAGO, CADO and carbachol on $DPSP_A$s. In (a) synaptic traces from left to right are $DPSP_A$s recorded in control medium, in the presence of 0.5 μM DAGO and in the combined presence of 0.5 μM DAGO and 10 μM naloxone. (b) shows $DPSP_A$s evoked in control medium, in the presence of 10 μM CADO and following a 20 min washout. (c) shows representative $DPSP_A$s evoked immediately before carbachol application, in the presence of 10 μM carbachol and in the combined presence of 10 μM carbachol and 5 μM atropine. The synaptic traces in (a), (b) and (c) were obtained from three separate neurones and in each case those traces recorded in the presence of drugs were obtained 20 min after the start of perfusion of the compound(s) specified. In (d) bar graphs compare the relative effects of DAGO, CADO and carbachol on the peak amplitude of $DPSP_A$s evoked by stimulation in either stratum oriens (left-hand graph) or stratum radiatum (right hand graph).

$IPSP_A$s ($n=3$) and $DPSP_A$s ($n=3$; Figure 1b), so identifying both responses as being mediated by the activation of $GABA_A$ receptors.

Presynaptic modulation of $DPSP_A$s

Activation of $GABA_B$, μ-opioid and muscarinic acetylcholine receptors on inhibitory interneurone terminals inhibits $IPSP_A$s through presynaptic inhibition of GABA release (Davies *et al*., 1990; Lambert *et al*., 1991b; Lambert & Teyler, 1991b; Cohen *et al*., 1992; Behrends & ten Bruggencate, 1993; Thompson *et al*., 1993) whereas activation of adenosine receptors has little effect (Lambert & Teyler, 1991a). As such, we addressed next whether the GABAergic afferents that were responsible for the generation of the $DPSP_A$ were subject to similar regulation by these receptor systems.

Qualitatively similar results were observed for each drug tested irrespective of whether synaptic responses were evoked by stimulation in stratum radiatum or stratum oriens. Therefore, all subsequent descriptions of data will simply refer to $IPSP_A$s or $DPSP_A$s without mention of the dendritic field stimulated. Precise quantification of the effects of each drug on each type of response evoked by stimulation in stratum oriens and stratum radiatum are provided in the Figures and accompanying legends.

Applications of 5 μM (−)-baclofen ($n=3$) caused reductions in cell input resistance (5–9%), postsynaptic hyperpolarizations (up to 4–5 mV from resting membrane potentials) and substantial depressions (up to 99%) of $DPSP_A$s that were completely reversed by CGP 55845A (1 μM, $n=3$, Figure 2a). In addition, in the combined presence of (−)-baclofen and CGP 55845A the amplitude and duration of the $DPSP_A$ was larger than in control medium (Figure 2a,c). We, therefore, tested the effect of CGP 55845A alone. This antagonist had no effect on the passive membrane properties of the cell but enhanced both the peak amplitude and duration of the $DPSP_A$ (Figure 2b,c).

We next tested the effects of the μ-opioid agonist DAGO and the non-hydrolyzable adenosine and acetylcholine analogues 2-chloroadenosine (CADO) and carbachol, respectively. In four cells DAGO, at a concentration 0.5 μM, had little effect on passive membrane properties but caused a substantial depression (up to 92%) of $DPSP_A$s that was readily reversed by subsequent application of naloxone (10 μM, $n=4$, Figure 3a). CADO, at 10 μM, caused a greater postsynaptic hyperpolarization and decrease in input resistance than (−)-baclofen (i.e. up to 17% and 12 mV (c.f. 9% and 5 mV) but only a relatively small depression of the $DPSP_A$ (Figure 3d) that was reversible on washout ($n=4$ for s. oriens and $n=5$ for s. radiatum; Figure 3b). In contrast, carbachol (10 μM) caused a postsynaptic depolarization (up to 17 mV) and an increase in cell input resistance (up to 30%). Despite this change in cell input resistance carbachol also caused a 40–50% depression of $DPSP_A$s ($n=6$ for s. oriens; $n=4$ for s. radiatum) that was reversed by the selective muscarinic acetylcholine receptor antagonist atropine (5 μM; Figure 3c).

Postsynaptic modulation of $IPSP_A$s and $DPSP_A$s

Having established that $DPSP_A$s were subject to regulation by those agonists that are known to presynaptically inhibit $IPSP_A$s we next addressed the issue of whether or not the

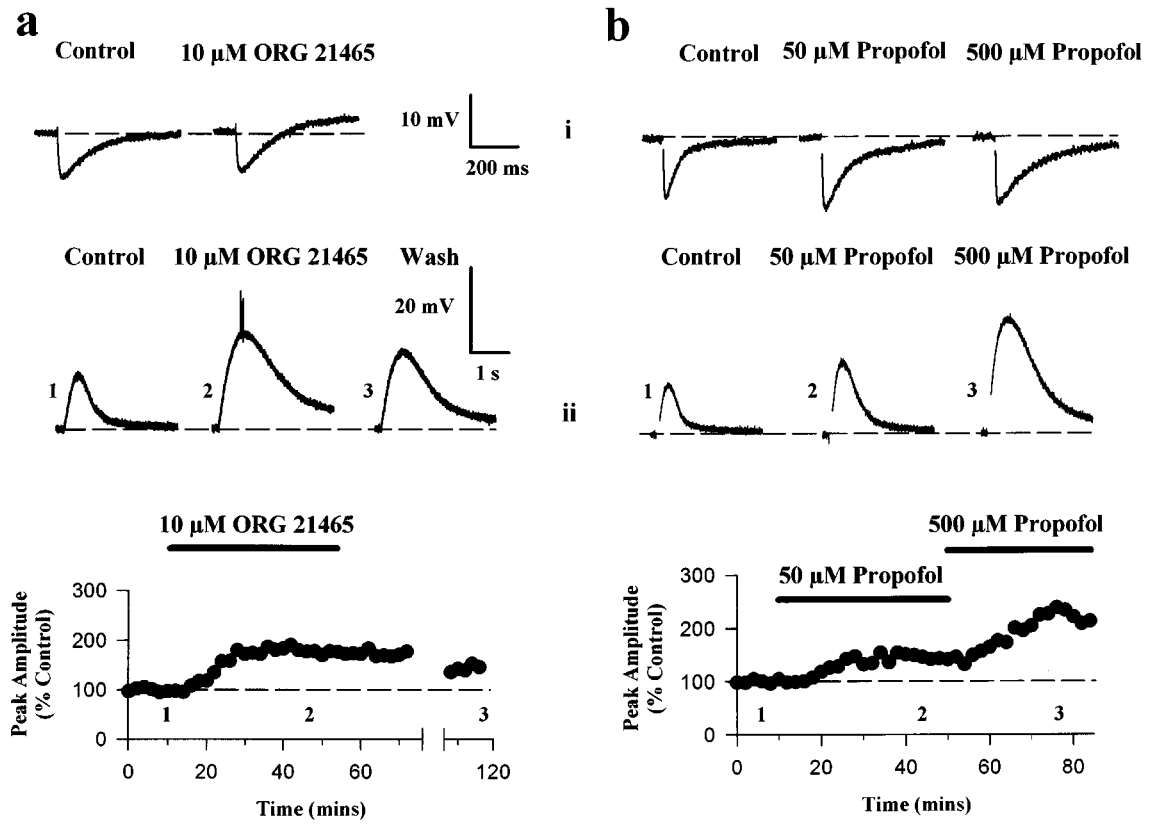

Figure 4 Effects of ORG 21465 and propofol on $IPSP_A$s and $DPSP_A$s. (a) and (b) illustrate the effects of 10 μM ORG 21465 and 50–500 μM propofol on $IPSP_A$s (i) and $DPSP_A$s (ii). Each set of synaptic traces is taken from a different neurone and the graphs under each set illustrate the time course of the facilitatory effects of the respective drugs on the $DPSP_A$. Note that ORG 21465 promoted the activation of the $DPSP_A$ sufficiently for it to cause the firing of two action potentials. These are truncated due to the averaging procedure and low sampling rate used to capture the $DPSP_A$.

pharmacology of the postsynaptic receptors that mediate $IPSP_As$ and $DPSP_As$ are similar. To do this we tested the effects of a number of known allosteric modulators of $GABA_A$ receptor function.

Neurosteroid, anaesthetic and barbiturate sites

In a first series of experiments we tested the effects of ligands that have been reported to potentiate $GABA_A$ receptor-mediated responses at low concentrations, directly activate $GABA_A$ receptors at high concentrations and which, in a variety of expression systems, have not been reported to differentiate significantly between $GABA_A$ receptors comprised of different subunit combinations (Sieghart, 1995; Lambert *et al.*, 1995). The ligands we chose to study were the water soluble neurosteroid ORG 21465, the intravenous anaesthetic propofol and the barbiturate pentobarbitone.

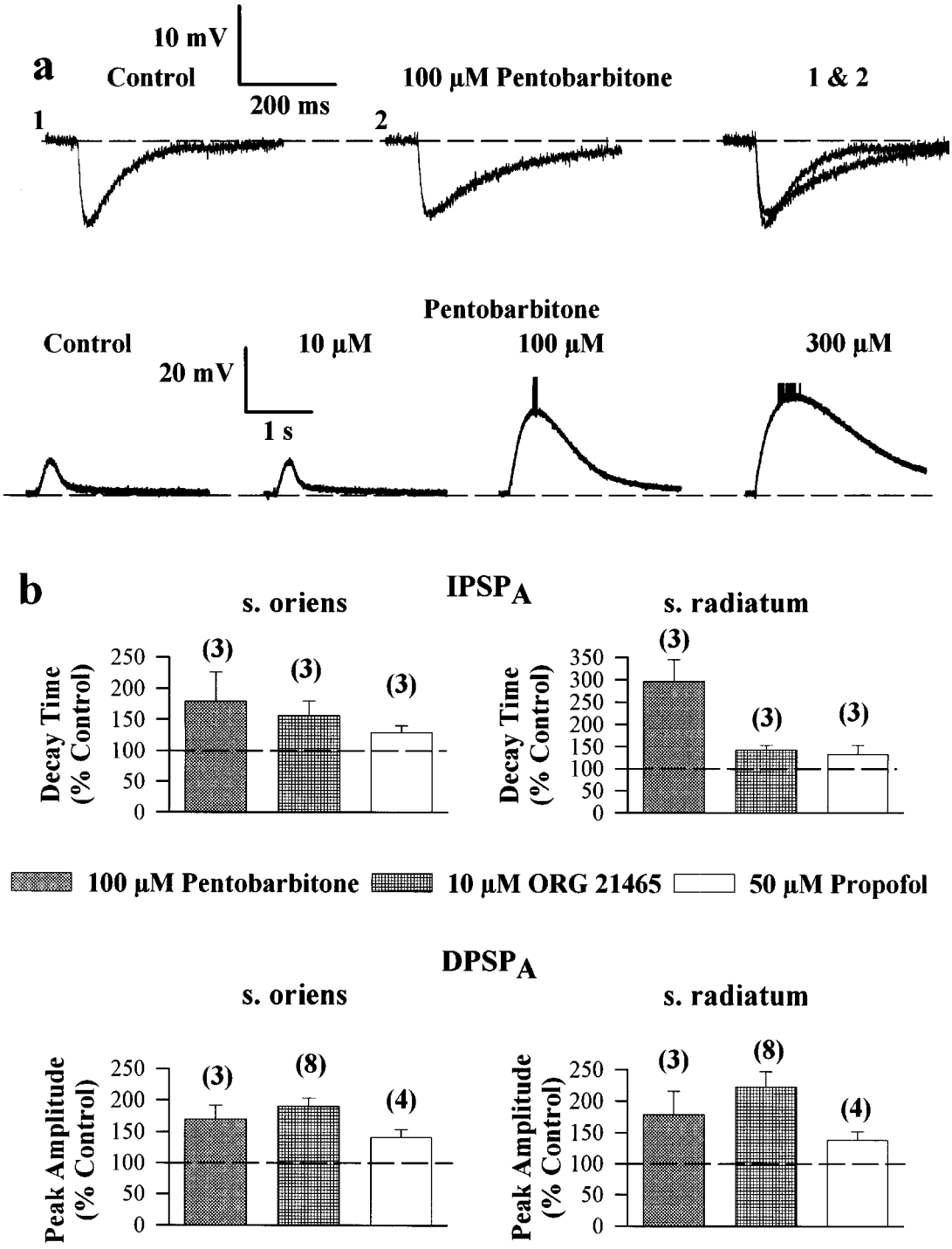

Figure 5 Comparison of the effects of pentobarbitone on $IPSP_As$ and $DPSP_As$ with those of ORG 21465 and propofol. (a) illustrates the effects of 100 μM pentobarbitone on the $IPSP_A$ (upper traces) and 100 μM and 300 μM pentobarbitone on the $DPSP_A$ (lower traces) in a different neurone. The bar graphs in (b) show pooled data for the effects of 100 μM pentobarbitone, 10 μM ORG 21465 and 50 μM propofol on the decay time constant of $IPSP_As$ and peak amplitude of $DPSP_As$ evoked by stimulation in stratum oriens and stratum radiatum. Note that the data for the effect of ORG 21465 on $IPSP_As$ were obtained from cells held at a membrane potential between -50 to -55 mV (see Results).

ORG 21465 had no effect on passive cell membrane properties but enhanced both the amplitude and the duration of the $DPSP_A$ in a concentration dependent and partially reversible manner. Thus, threshold effects were observed at a concentration of 1 μM and maximal potentiation observed at 10 μM ($n=8$). In contrast, ORG 21465 appeared to reduce slightly the amplitude of the $IPSP_A$ in some instances. However, this reduction was a consequence of increased activation of the $DPSP_A$ superimposed on the $IPSP_A$ (Figure 4a) because when the cell was held at a membrane potential of -50 to -55 mV, at which the $DPSP_A$ was negligible, ORG 21465 had a small potentiating effect on the peak amplitude of the $IPSP_A$ that was associated with a substantial increase in its duration (Figure 5b).

Like ORG 21465, propofol (50 μM) and pentobarbitone (100 μM) had no overall effect on passive membrane properties

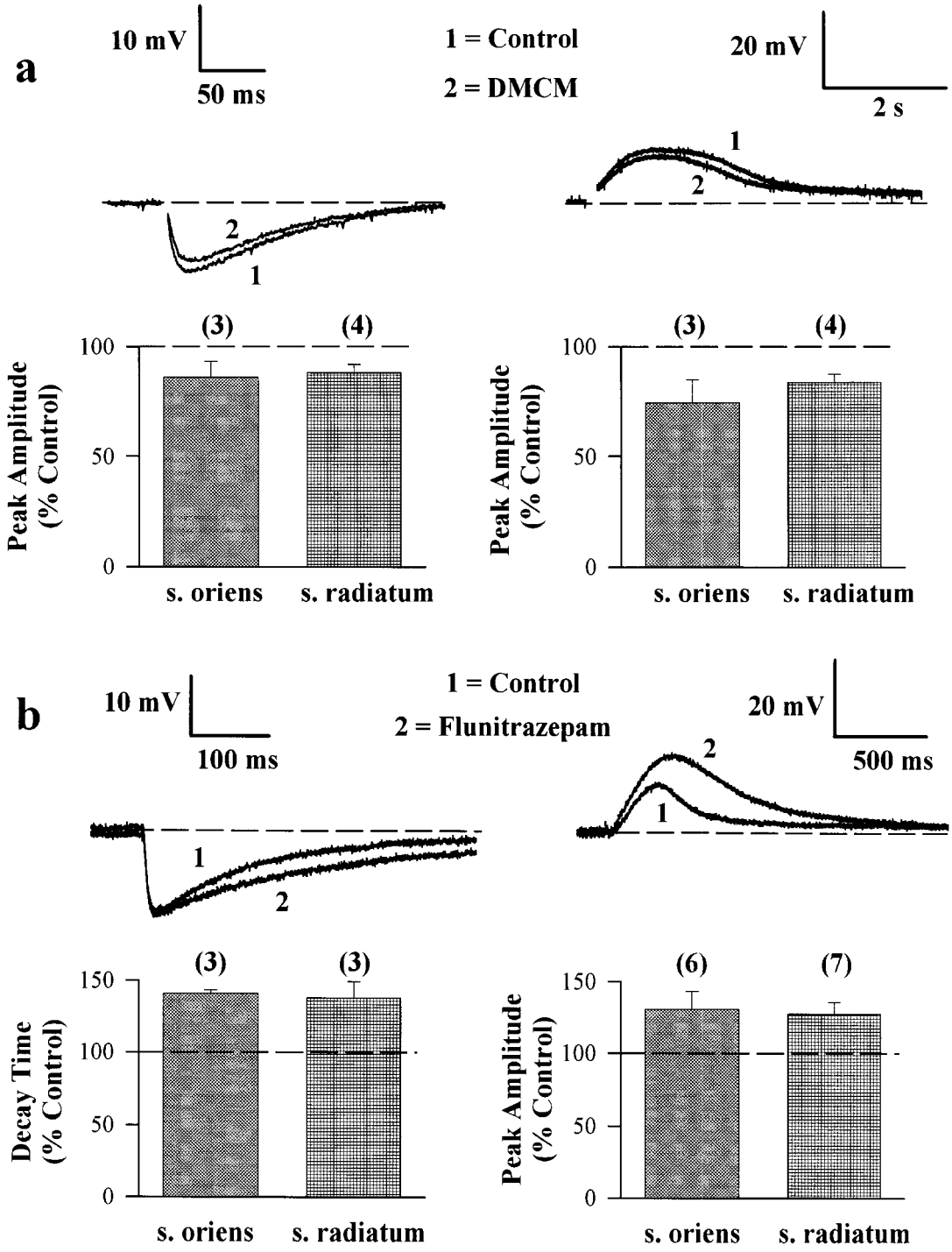

Figure 6 Effects of the benzodiazepine site ligands DMCM and flunitrazepam on $IPSP_A$s and $DPSP_A$s. In (a) left-hand traces represent superimposed $IPSP_A$s evoked in the presence and absence of 1 μM DMCM. Right-hand traces represent $DPSP_A$s under the same conditions obtained from a different neurone. The bar graphs below each set of synaptic traces illustrate pooled data for the effect of 1 μM DMCM on the decay time constant of $IPSP_A$s and the peak amplitude of $DPSP_A$s evoked by stimulation in either stratum oriens or stratum radiatum. (b) is a similar format to (a) and illustrates the potentiation of $IPSP_A$s (left hand traces and bar graph) and $DPSP_A$s (right-hand traces and bar graph) by 50 μM and 10 μM flunitrazepam, respectively.

of cells but enhanced the duration of $IPSP_As$ ($n=3$ for each drug; Figures 4b and 5, respectively). In addition, both drugs increased the amplitude and duration of $DPSP_As$ ($n=4$; Figures 4b and 5). These effects were concentration dependent. Thus, 50 μM propofol produced only 40–50% of the level of potentiation evoked by a concentration of 500 μM (Figure 4b). In the case of pentobarbitone, a concentration of 10 μM was just suprathreshold for enhancing both $IPSP_As$ and $DPSP_As$

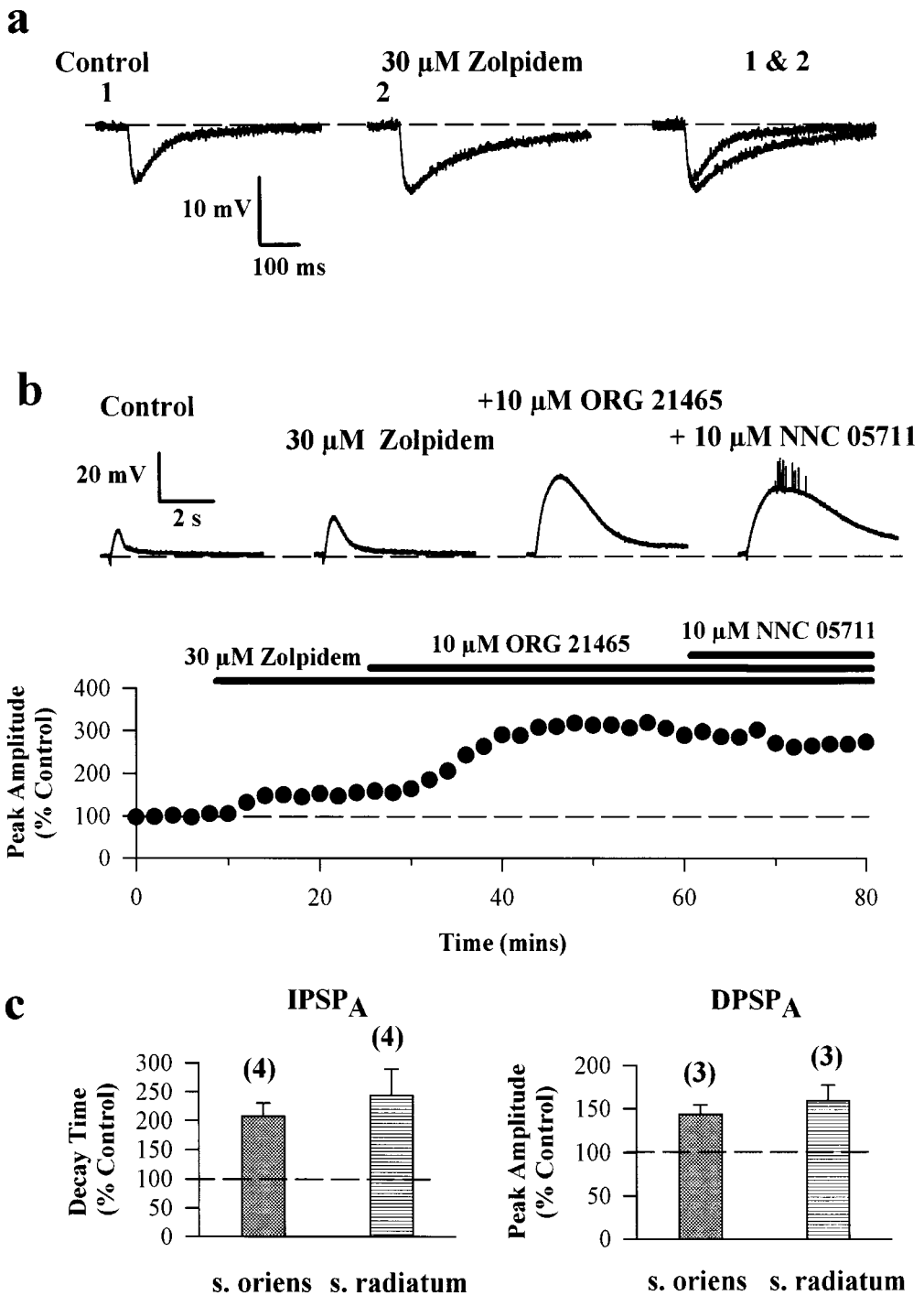

Figure 7 Zolpidem facilitates both $IPSP_As$ and $DPSP_As$. In (a) the left and middle traces are $IPSP_As$ recorded immediately prior to zolpidem application (1) and after 20 min in the presence of 30 μM zolpidem (2), respectively. The right-hand traces show these two $IPSP_As$ superimposed (1 and 2) to illustrate the level of potentiation induced by zolpidem. In (b) the synaptic traces, from left to right, are $DPSP_As$ recorded from a separate neurone just prior to zolpidem application, in the presence of 30 μM zolpidem, in the combined presence of 30 μM zolpidem and 10 μM ORG 21465, and in the combined presence of 30 μM zolpidem, 10 μM ORG 21465 and 10 μM NNC 05-711. Note that in the additional presence of NNC 05-711 the $DPSP_A$ was sufficiently large to generate the firing of a burst of action potentials which are truncated due to trace averaging and the low sampling rate required to capture the $DPSP_A$. The graph below these synaptic traces plots the peak amplitude of the $DPSP_A$ throughout the course of this experiment expressed as a percentage of the mean peak amplitude of the five $DPSP_As$ evoked prior to the application of zolpidem. In (c) the bar graphs show pooled data for the effects of 30 μM zolpidem on the decay time constant of $IPSP_As$ and peak amplitude of $DPSP_As$ evoked by stimulation in either stratum radiatum or stratum oriens.

and a concentration of 100 μM near maximal for potentiating the peak amplitude of $DPSP_A$s such that increasing the concentration to 300 μM produced only a small change in this parameter but greatly prolonged the duration of the $DPSP_A$ (Figure 5a).

Benzodiazepine site

To extend the analysis of allosteric modulation of the $IPSP_A$ and the $DPSP_A$ the effects of a range of benzodiazepine (BZ) site ligands on these responses were tested. The rationale behind these experiments was to gain some insight into the subunit composition of the $GABA_A$ receptors mediating these responses using BZ ligands because these compounds exhibit differential preferences for $GABA_A$ receptors comprised of specific subunits (Sieghart, 1995). Initially we tested the effects of the $GABA_A$ receptor BZ site inverse agonist DMCM (1 μM) which differentially modulates the activity of a wide range of hetero-oligomeric $GABA_A$ receptors. This compound had no effect on cell membrane potential or input resistance but produced a modest but consistent depression of both the peak amplitude of the $DPSP_A$ (up to 26%) and the $IPSP_A$ (up to 42%) (Figure 6a). In contrast, the BZ site agonist flunitrazepam at 50 μM prolonged the $IPSP_A$ (by up to 59%; $n=3$) and at 10 μM increased both the amplitude (up to 30%) and the duration of the $DPSP_A$ ($n=6-7$; Figure 6b). No effects on passive membrane properties of cells were observed.

Having established that relatively broad spectrum benzodiazepine site ligands could positively or negatively affect the $DPSP_A$, we addressed next whether zolpidem, which exhibits selectivity for $BZ\omega_1$ sites over $BZ\omega_2$ sites (Sieghart, 1995), also affected the $IPSP_A$ and the $DPSP_A$. As illustrated in Figure 7, zolpidem (30 μM) had similar effects to those observed for flunitrazepam (Figure 6b). Thus, at the lowest concentration (1 μM) tested zolpidem had little or no effect on the $IPSP_A$ or the $DPSP_A$. However, at 10 μM it induced a 20% (not illustrated), and at 30 μM a 50%, increase in the peak amplitude of the $DPSP_A$ (Figure 7b,c). Similarly, zolpidem (30 μM) increased the decay time constant of the $IPSP_A$ ($n=4$; Figure 7a,c). Subsequent addition of 10 μM ORG 21465 produced an additional enhancement of both the amplitude and the duration of the $DPSP_A$ indicating there was additional capacity to enhance this response. No further increase in amplitude was observed when 10 μM NNC 05-711 was subsequently added although the duration of the response was substantially prolonged. This ensured that the $DPSP_A$, in the presence of the three drugs, was now sufficiently large to trigger the firing of a burst of action potentials (Figure 7b).

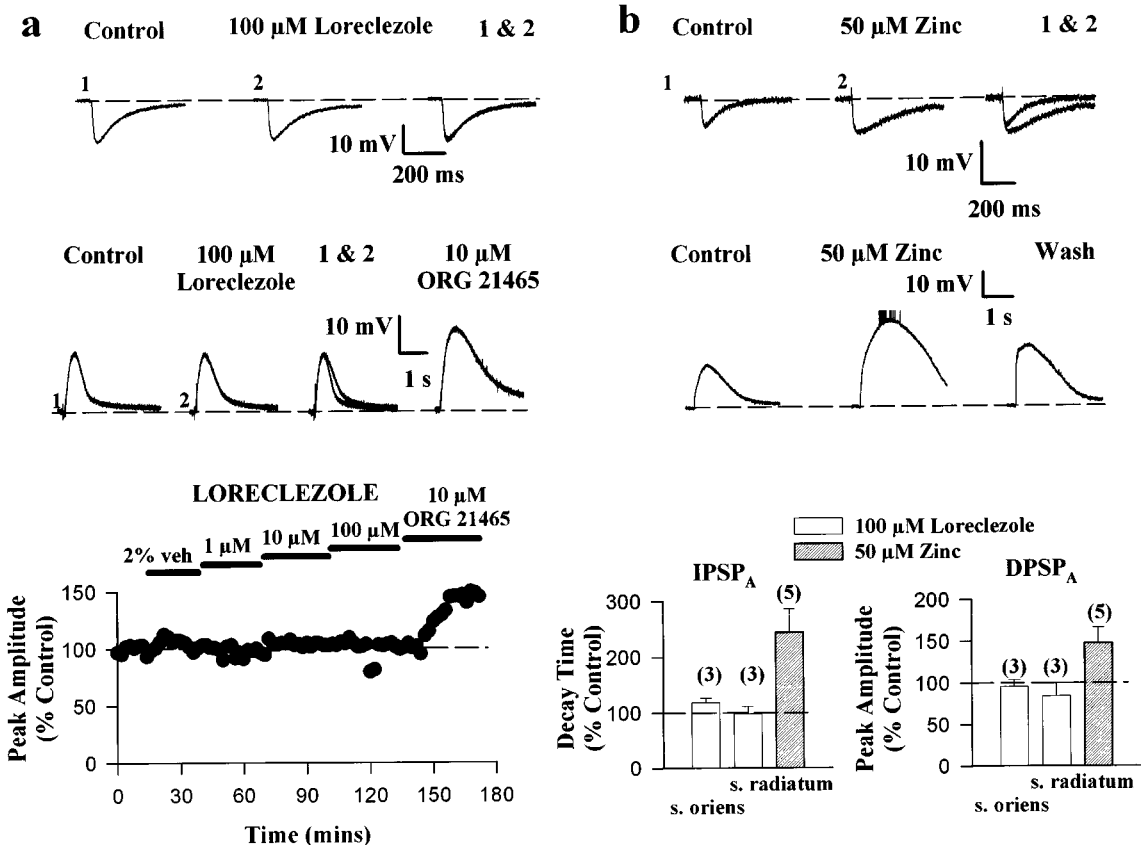

Figure 8 Effects of loreclezole and zinc on $IPSP_A$s and $DPSP_A$s. In (a) upper traces are $IPSP_A$s. The left-hand and middle traces represent $IPSP_A$s evoked in the absence (1) and presence (2) of 100 μM loreclezole. The right-hand trace shows these synaptic responses superimposed to illustrate the lack of effect of loreclezole. Synaptic traces below these three, from left to right, represent $DPSP_A$s in control medium, (1), in the presence of 100 μM loreclezole (2) and the superimposition of these two traces (1 and 2). The extreme right-hand trace shows the subsequent increase in the $DPSP_A$ amplitude evoked by subsequent application of 10 μM ORG 21465. Note that although no change in the peak amplitude of the $DPSP_A$ was observed, loreclezole caused a slight increase in the duration of this response. The graph below the traces plots the peak amplitude of the $DPSP_A$ throughout the course of the experiment. In (b) upper traces from left to right are $IPSP_A$s evoked in control medium (1), in the presence of 50 μM zinc (2) and these two responses superimposed (1 and 2). Synaptic traces below these illustrate $DPSP_A$s evoked in control medium, in the presence of 50 μM zinc and following 30 min washout. Note that zinc enhanced both $IPSP_A$s and $DPSP_A$s. The bar graphs show pooled data for the effects of 100 μM loreclezole and 50 μM zinc on the decay time constant of $IPSP_A$s and peak amplitude of $DPSP_A$s evoked by stimulation in either stratum radiatum or stratum oriens.

The anticonvulsant loreclezole

To provide further insight into the subunit composition of the $GABA_A$ receptors mediating the $IPSP_A$ and the $DPSP_A$ the effects of the anticonvulsant agent loreclezole on these responses were assessed. This compound was chosen becaues it discriminates between $GABA_A$ receptors containing $\beta 2/3$ subunits from those containing $\beta 1$ subunits (Wingrove *et al*., 1994). Loreclezole (1 – 100 μM) had minimal effects on cell membrane potential, input resistance or peak amplitude and decay time constants of $IPSP_A$s ($n = 3$; Figure 8a,c). Likewise, it did not consistently affect the amplitude of the $DPSP_A$. However, in three out of four cells it appeared to prolong slightly the $DPSP_A$ (Figure 8b). In these cells, subsequent application of ORG 21465 substantially enhanced the amplitude and duration of the $DPSP_A$ (Figure 8b) indicating that these responses were still susceptible to modulation by other allosteric modulators of $GABA_A$ receptor function.

Zinc

To complete the analysis of allosteric modulation of the $IPSP_A$ and the $DPSP_A$ the effects of zinc were tested since this divalent cation selectively antagonizes those $GABA_A$ receptors which are devoid of γ-subunits (Draguhn *et al*., 1990; Smart *et al*., 1991). Zinc (50 μM) had no substantial effect on cell membrane potential or input resistance but produced a consistent increase in both the peak amplitude and duration of the $DPSP_A$ (up to 205%). In addition, zinc produced a substantial increase in the decay time of the $IPSP_A$ (up to 387%) which was associated with a small increase in the peak amplitude of this response (Figure 8). The paradoxical effects of zinc on both responses likely reflect actions of this cation on components of GABA-mediated synaptic transmission other than the $GABA_A$ receptor itself, e.g., K^+ conductances regulating GABA release (Xie & Smart, 1993).

Discussion

$GABA_A$ receptors mediate the $IPSP_A$ and the $DPSP_A$

The enhancement by NNC 05-711, and depression by bicuculline, of both the $IPSP_A$ and the $DPSP_A$ are entirely consistent with both responses being mediated by GABA activation of $GABA_A$ receptors; a conclusion in agreement with a number of other reports in which a variety of experimental protocols have been employed to activate these responses (Alger & Nicoll, 1982a,b; Xie & Smart, 1993; Grover *et al*., 1993; Davies & Collingridge, 1993; Taira *et al*., 1997). In addition, the effect of NNC 05-711 to prolong the $IPSP_A$ without significantly affecting its amplitude is consistent with the effects of the structurally related GABA uptake blocker tiagabine (Roepstorff & Lambert, 1992,1994; Thompson & Gähwiler, 1992). Together these data suggest that synaptically released GABA initially produces a high degree of saturation of synaptic $GABA_A$ receptors and that the enhanced duration of the $IPSP_A$ and the potentiation of the $DPSP_A$ are accounted for by the increased persistence of GABA in the synaptic cleft.

Origin of the $DPSP_A$

The major source of GABA-mediated synaptic transmission in the hippocampus derives from GABAergic interneurones. Within the CA1 region there are numerous interneurone subtypes (e.g., axo-axonic, basket cells (Buhl *et al*., 1994)) that are differentially regulated by presynaptic heteroreceptors or $GABA_B$ autoreceptors (Freund & Buszáki, 1996). Here, although we cannot discriminate between which populations of interneurones are, or are not, activated by the stimulation protocols used it is quite clear that $DPSP_A$s, like $IPSP_A$s, are inhibited by activation of $GABA_B$ and μ-opioid receptors. In fact, the degree of inhibition caused by the agonist concentrations used in this study closely mirrors that reported for $IPSP_A$s in previous studies (Davies *et al*., 1990; Lambert *et al*., 1991b; Pearce *et al*., 1995). In these studies, it was demonstrated that the predominant action of these drugs to reduce the $IPSP_A$s was *via* presynaptic inhibition of release rather than *via* the associated decrease in input resistance and consequent postsynaptic shunting of synaptic responses. In this respect, the relatively small inhibitory influence afforded by adenosine receptors is most likely explained on the basis of changes in cell input resistance as CADO has little effect on $IPSP_A$s (Lambert & Teyler, 1991a), indicating the relative absence of adenosine A_1 heteroceptors on inhibitory interneurone terminals. In contrast, mAChRs have been shown using immunocytochemical methods to be present on the terminals of certain populations of interneurones (Hájos *et al*., 1998). However, conflicting reports exist concerning the effect of activation of mAChRs on $IPSP_A$s. Thus, carbachol has been reported to have a depressant action (Behrends & ten Bruggencate, 1993) or no effect (Lambert & Teyler, 1991b) on the $IPSP_A$. In the present study, we observed a moderate depressant action of carbachol on $DPSP_A$s that was similar in magnitude to that reported by Behrends & ten Bruggencate (1993) on $IPSP_A$s. However, carbachol, even at 10 μM, never abolished $DPSP_A$s suggesting either that mAChRs regulate only select populations of interneurones, which supports the immunocytochemical data (Hájos *et al*., 1998), or that these receptors are less effective than μ-opioid or $GABA_B$ receptors at inhibiting the release of GABA. Whatever the case, these data taken together with that for DAGO and (−)-baclofen suggest that interneurones that mediate the $IPSP_A$ and the $DPSP_A$ are subject to similar presynaptic regulation by $GABA_B$ autoreceptors as well as μ-opioid and mACh heteroreceptors. The slight differences in sensitivity of stratum oriens- *versus* stratum radiatum-evoked $DPSP_A$s to (−)-baclofen but not DAGO correlates well with that reported for $IPSP_A$s (Pearce *et al*., 1995; Lambert & Wilson, 1993) supporting the suggestion that differential expression of presynaptic receptors by separate interneurone types exists in these dendritic fields. That CGP 55845A alone enhanced the $DPSP_A$ may reflect antagonism of $GABA_B$ autoreceptors that are activated by endogenously released GABA during repetitive stimulation. However, part of this effect also likely reflects the block of a $GABA_B$ receptor-mediated IPSP that is superimposed on the $DPSP_A$.

In summary, therefore, the simplest explanation for why mAChR, $GABA_B$, μ-opioid and adenosine receptors exhibit a similar pattern of inhibition of $IPSP_A$s to that observed for $DPSP_A$s is that the same populations of interneurones, in a given dendritic field, mediate both responses. In this respect, it is worth noting that stimulation in each dendritic field is likely to activate different types of interneurones. For example, stimulation in stratum oriens is likely to incorporate a contribution from horizontal trilaminar cells and oriens-lacunosum interneurones (Lambert *et al*., 1991a; Freund & Buzsáki, 1996) which is likely to be reduced or absent following stimulation in stratum radiatum. As such, it is likely that all interneurone types are capable of activating $DPSP_A$s as well as $IPSP_A$s. That said, it is still conceivable that high frequency stimulation may recruit different interneurone

type(s) which selectively mediate the $DPSP_A$ and in which the release of GABA is regulated similarly to those mediating the $IPSP_A$. This, however, is unlikely since the same single shock stimulation strength that evokes a pure $IPSP_A$ in control medium evokes an $IPSP_A$ followed by a $DPSP_A$ in the presence of ORG 21465 at resting membrane potentials.

Similar $GABA_A$ receptors mediate $IPSP_A$s and $DPSP_A$s

At the postsynaptic level this study has directly compared the effects of a range of allosteric modulators of $GABA_A$ receptor function on the $IPSP_A$ and the $DPSP_A$ in age-matched animals and often on the same neurone to eliminate possible developmental/hormonal changes in the effectiveness of each allosteric modulator (Rovira & Ben-Ari, 1993; Brussaard *et al*., 1997). Based on the data generated for a wide range of allosteric modulators of $GABA_A$ receptor function, it was not possible to differentiate pharmacologically between those receptors that mediate the $IPSP_A$ and those the $DPSP_A$. That is not to say that subtle pharmacological differences do not exist in the receptors mediating each type of synaptic response. However, any attempt to establish such subtle quantitative differences is complicated for numerous reasons. One problem is that different methodologies are required to activate each response i.e. high frequency burst for $DPSP_A$s and single stimulus for $IPSP_A$s. These paradigms unavoidably produce distinct transients of GABA release (i.e. pulsatile release for $DPSP_A$s that lasts almost the entire duration of the $IPSP_A$ evoked by a single pulse of GABA). The consequence of this is that for $DPSP_A$s all released GABA barring the initial pulse will encounter populations of receptors in different conformational states (e.g., uni-liganded, closed, desensitized) to those which generate $IPSP_A$s because of their prior history of activation. In turn this may lead to apparent differences in allosteric modulation that do not, in their own right, correspond to molecular differences in the $GABA_A$ receptors mediating the two responses. In addition, the current uncertainty of the ionic mechanisms underlying each response complicates quantitative interpretation of pharmacological data because in the case of the Staley identical receptor ionic gradient dissipation model $DPSP_A$s are pre-disposed to greater sensitivity to antagonism and allosteric modulation than are $IPSP_A$s. As such, here we have provided quantified pooled data simply as a measure of the effect of each drug on each type of response without any attempt to make direct comparisons between responses. That said, what is clear is that both synaptic responses are subject to allosteric modulation by a common range of pharmacological agents. In this respect, the effects of zolpidem, DMCM and pentobarbitone on the $IPSP_A$ qualitatively support those reported previously (Alger & Nicoll, 1982a,b; Rovira & Ben-Ari, 1993). In addition, the effects of pentobarbitone on the $DPSP_A$ are consistent with those observed by Alger & Nicoll (1982a,b). However, unlike the present study, a measurable enhancement of the $DPSP_A$ by benzodiazepine ligands was not observed by these investigators. One explanation for this discrepancy may be that in the original study $DPSP_A$s were evoked in conjunction with $IPSP_A$s and, as such, any enhancement of the $DPSP_A$ may have been masked by a facilitatory effect on the $IPSP_A$. The data presented here extends the pharmacological evaluation of synaptically activated $GABA_A$ receptors and demonstrates that propofol, flunitrazepam and ORG 21465 have positive allosteric effects on both the $IPSP_A$ and the $DPSP_A$ whereas loreclezole is largely ineffective barring the slight prolongation of the $DPSP_A$ in three out of four neurones. In addition, the threshold and maximal concentrations of each drug tested were equivalent for both $IPSP_A$s and $DPSP_A$s suggesting that the receptors mediating both responses are similarly affected by each agent. In this respect, it is quite clear that both the $IPSP_A$ and the $DPSP_A$ are modulated by (1) barbiturates, (2) neurosteroids, (3) anaesthetics, (4) benzodiazepine site inverse agonists, and (5) benzodiazepine site full agonists irrespective of whether the ligand has a benzodiazepine or non-benzodiazepine structure (e.g., the imidazopyridine zolpidem). The enhancement of both responses by zinc is consistent with previous reports but contradicts its known negative allosteric modulatory effect on certain $GABA_A$ receptors (Draguhn *et al*., 1990; Xie & Smart, 1991). As such, the effect of this divalent cation likely results from interactions with other mechanisms involved in the generation of both $DPSP_A$s and $IPSP_A$s. Whether these be at a pre- or post-synaptic level or both is unclear. Whatever the case, that zinc affects $IPSP_A$s and $DPSP_A$s in a similar manner re-emphasizes the commonality of mechanisms involved in the transmission of $IPSP_A$s and $DPSP_A$s.

The question arises, however, as to which of the multiple $GABA_A$ receptor subunits, expressed in rodent brain, form the receptors mediating $IPSP_A$s and $DPSP_A$s. To date, immunoprecipitation studies in the mammalian brain have identified the expression of at least 14 subunits (excluding splice variant forms). These include six α (termed $\alpha 1-6$), three β (termed $\beta 1-3$), three γ (referred to as $\gamma 1-3$), one δ and one ε form (Wisden & Seeburg, 1992; MacDonald & Olsen, 1994). Harmonic analysis of electron microscopic images of porcine cortical $GABA_A$ receptors combined with co-precipitation techniques, immunofluorescent staining and *in situ* hybridization, have suggested further that each $GABA_A$ receptor is a pentameric hetero-oligomer in which the likely stoichiometry of subunits is $(\alpha)_2\beta(\gamma)_2$ (Barnard, 1996). Within this array the α subunits need not be identical and one or both of the γ subunits can be replaced by the δ or ε subunit. Based on this configuration, the possible diversity of receptor combinations, even taking into account forbidden hetero-oligomeric combinations, runs into tens of receptors.

With respect to the $GABA_A$ receptor-mediated synaptic responses studied here the effectiveness of bicuculline and picrotoxin to block both $IPSP_A$s and $DPSP_A$s (Davies *et al*., 1990; Davies & Collingridge, 1993) provides little insight into the subunit composition of the receptors mediating these responses since these agents do not readily discriminate between different hetero-oligomeric $GABA_A$ receptor complexes (Krishek *et al*., 1996). Likewise, immunoprecipitation (McKernan & Whiting, 1996) and *in situ* hybridization (Wisden *et al*., 1991) studies performed in the hippocampus have provided few clues to the identity of the $GABA_A$ receptor(s) mediating the $IPSP_A$ and $DPSP_A$ since all currently identified $GABA_A$ receptor subunits, except the $\alpha 6$ subunit, are expressed in this area of the brain. However, the formation of certain hetero-oligomeric receptor combinations appear to be preferred. In particular, $\alpha 5\beta 3\gamma 2/3$, $\alpha 2\beta x\gamma 2$ and $\alpha 1\beta 2\gamma 2$ combinations are highly expressed. An immunocytochemical investigation of the distribution of $\alpha 1$ and $\alpha 2$ subunits in CA1 pyramidal neurones has demonstrated that $\alpha 1$ subunits are expressed throughout the somato-dendritic axis, at locations that correlate with synaptic contacts made by a variety of GABAergic interneurone types, whereas $\alpha 2$ are expressed almost exclusively at the axon initial segment, at sites of synaptic contact presumed to be made by axo-axonic cells (Nusser *et al*., 1996). It is surprising, therefore, that in the present study, $IPSP_A$s and $DPSP_A$s in pyramidal neurones were relatively insensitive to zolpidem suggesting the absence of an $\alpha 1$ subunit in the receptors mediating these responses.

However, it is plausible that the assembly of other α subunit isoforms and/or the γ subunit with the α1 subunit in the native hetero-oligomeric receptor complexes may reduce BZ site sensitivity. In terms of the other α subunits expressed in the hippocampus, α5 subunits are the most abundant (McKernan *et al*., 1991), α4 subunits are not recognized by flunitrazepam (Wisden *et al*., 1991) and α3-containing receptors exhibit the slowest kinetics (Gingrich *et al*., 1995). Based on the latter observation it is tempting to speculate that α3-containing receptors are responsible for mediating the $DPSP_A$. However, comparison of the effective concentrations of zolpidem, DMCM and flunitrazepam here with K_i values reported for numerous hetero-oligomeric recombinant receptors (Pritchett & Seeburg, 1990; Hadingham *et al*., 1993; Ymer *et al*., 1990) does not readily reveal the identity of the receptors mediating $IPSP_A$s and $DPSP_A$s. That both responses are susceptible to benzodiazepine site ligands, and not inhibited by zinc, indicates the presence of a γ subunit. The weak effect of loreclezole on both $IPSP_A$s and $DPSP_A$s argues against the inclusion of either β2 or β3 isoforms in the receptors mediating these synaptic responses although it may be that coexpresion of different combinations of α, γ and δ subunits with β2/3 subunits might adversely affect the potency of this compound. However, δ subunits are unlikely to be present in the receptors under study here since the inclusion of this subunit in the $GABA_A$ receptor confers reduced potency to neurosteroids (Zhu *et al*., 1996) which was not the case for either $IPSP_A$s or $DPSP_A$s. Likewise, whilst positive allosteric modulation by propofol or pentobarbitone might indicate the absence of the ε subunit in receptors mediating these responses the concept that this subunit prevents anaesthetic sensitivity (Davies *et al*., 1997) has recently been questioned (Whiting *et al*., 1997).

In summary, this study provides little evidence to support the hypothesis that activation of different $GABA_A$ receptors (Perkins & Wong, 1996), possibly activated by different types of interneurone, are responsible for the generation of the $IPSP_A$ and the $DPSP_A$. In this respect, we have demonstrated that both responses are subject to common modulation by a number of presynaptic heteroreceptors/autoreceptors and a wide range of postsynaptic allosteric modulators. Based on a comparison of these data with that published for $GABA_A$ receptors expressed in numerous cell lines it is not possible to establish the exact make-up of the receptors mediating the $DPSP_A$ and the $IPSP_A$. Unequivocal determination of the precise $GABA_A$ receptor subunit combination mediating both responses awaits the development of ligands that are highly selective for receptors containing specific isoforms of each family of subunits.

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