Brain Research, 623 (1993) 329–333

BRES 25832

The progesterone metabolite 5α-pregnan-3α-ol-20-one reduces K^+-induced GABA and glutamate release from identified nerve terminals in rat hippocampus: a semiquantitative immunocytochemical study

Erik Taubøll [a], Ole Petter Ottersen [b] and Leif Gjerstad [a]

[a] *Department of Neurology, Rikshospitalet, The National Hospital, University of Oslo, Oslo (Norway) and* [b] *Department of Anatomy, University of Oslo, Oslo (Norway)*

(Accepted 22 June 1993)

Key words: 5α-Pregnan-3α-ol-20-one; Progesterone; Anticonvulsant; Epilepsy; Phenobarbital; Immunocytochemistry

5α-Pregnan-3αol-20-one (3α-OH-DHP) reduced the depolarization-induced loss of GABA and, to a lesser extent, the glutamate-like immunoreactivities from nerve terminals in the in vitro hippocampal slice. Phenobarbital (PB) had similar effects. These results suggest that 3α-OH-DHP affects presynaptic transmitter release, possibly in a barbiturate-like manner.

Progesterone, and especially its pregnanolone metabolites, exert strong anticonvulsant effects[1,10,19] of potential therapeutic value[5]. Progesterone and its metabolites act as barbiturate-like ligands of the GABA receptor complex and enhance GABA-ergic inhibition [8,12]. Both barbiturates[13] and progesterone[17] suppress excitatory responses to glutamate.

Presynaptically, barbiturates have been shown to inhibit Ca^{2+} uptake[18], and to reduce synaptosomal release of neurotransmitters including GABA and glutamate[4]. Possible presynaptic effects of progesterone metabolites have received less attention. A few biochemical studies indicate that progesterone reduces Ca^{2+}-uptake[14], and that it also may modify transmitter release[3,6]. In addition, a recent in vivo neurophysiological study in cat visual cortex suggests that 5α-pregnan-3α-ol-20-one (3α-OH-DHP) may exert presynaptic effects, possibly mediated through a reduced release of transmitter[22].

The aim of the present study was to investigate possible presynaptic actions of 3α-OH-DHP on K^+-induced release of GABA and glutamate from identified nerve terminals. PB is known to affect transmitter release and served as a reference substance. The CA1 subfield of hippocampus was examined since both drugs have been demonstrated to have marked neurophysiological effects in this area[21]. The necessary anatomical resolution was achieved by using an electron microscopic, immunocytochemical technique with colloidal gold particles as markers. This approach allows a semiquantitative assessment of the depolarization-induced depletion of glutamate and GABA from nerve endings[16].

Adult female Sprague–Dawley rats (Møllegaard, Skensved, Denmark) were decapitated during light ether anaesthesia. To minimize possible variations between different phases of the estrous cycle, all animals were used on the day of proestrous, according to vaginal smears. From each animal, consecutive hippocampal slices (450 μm) from one hippocampus were distributed sequentially in 2 separate brain slice chambers (BSC-PC, Medical Systems Corp.) containing arti-

Correspondence: E. Taubøll, Department of Neurology, Rikshospitalet, 0027 Oslo 1, Norway. Fax: (47) 22 868 890.

ficial cerebrospinal fluid (ACSF) of the following composition (mM): Na^+ 151, K^+ 5, Mg^{2+} 2, Ca^{2+} 2, Cl^- 133, HCO_3^- 26, SO_4^{2-} 2, $H_2PO_4^-$ 1, and glucose 10. The ACSF was bubbled through by 95% O_2/5% CO_2 and kept at 36°C.

After 1 hour, the solutions in the chambers were changed to ACSF containing 300 μM of 3α-OH-DHP or 8.6 mM of PB (chamber 1), or equivalent solutions without drugs (chamber 2). After 30 min [K^+] in both chambers was raised from 5 to 35 mM with a corresponding reduction of [Na^+] to maintain iso-osmolarity. The incubation was then continued for 20 min. For each drug/transmitter combination, at least one control experiment was performed with a third chamber containing consecutive slices from the same hippocampus. These were incubated in the vehicle solutions for the respective drugs, without addition of 35 mM K^+.

Solutions of 3α-OH-DHP (Sigma) were made by dissolving 5 mg of the substance in 0.5 ml of propyleneglycol. This was further diluted in 2 ml of 20% albumin in H_2O and then dripped slowly into 50 ml of ACSF (35°C), giving a final concentration of 300 μM. PB was dissolved in distilled water in a concentration of 100 mg/ml, and further diluted in ACSF to a final concentration of 8.6 mM. The concentration ratio between 3α-OH-DHP and PB used in the present study reflected their relative potencies in experiments designed to test their effect on cortical inhibition[21].

After incubation, the slices were immediately fixed by 2.5% glutaraldehyde (G) and 1% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (1 h). Following fixation, the slices were treated with OsO_4, dehydrated and embedded in an epoxy resin (for details, see ref. 15). Ultrathin sections through hippocampal field CA1 were treated as described[15]. The primary rabbit antisera were: glutamate antiserum 13 (1:300 or 1:500) or GABA antiserum 26 (1:75 or 1:100). The bound antibodies were visualized by sheep anti-rabbit IgG coupled to colloidal gold particles (GAR 15; Janssen, Beerse, Belgium). The antisera were raised according to the procedure of Storm-Mathisen et al.[20]; they have been thoroughly characterized and found to be highly selective after appropriate preadsorption[15,20]. The high selectivity was confirmed in the present experiments by use of test conjugates[15], and blocking of staining by preadsorptions with the respective antigens (glutaraldehyde complexes of glutamate or GABA). Sections to be compared were incubated simultaneously and in the same solutions of immunoreagents. This was a necessary precaution, due to the considerable inter-experimental variation in the absolute gold particle densities.

Two populations of nerve terminals in CA1 stratum oriens and stratum radiatum were analysed; (1) terminals establishing symmetric contacts with somata or proximal dendritic stems of pyramidal cells (putative GABA-ergic terminals, most of which belong to local interneurons), and (2) terminals establishing asymmetric contacts with spines of proximal pyramidal cell dendrites (putative glutamatergic terminals of the Schaffer collateral/commissural system). The former population was investigated with respect to immunolabelling intensity for GABA, and the latter for glutamate. Gold particle densities over the different nerve terminal types were assessed by means of a previously published procedure[2].

The data were analysed with BMDP 3V (general mixed model analysis of variance; Statistical Software Inc., Los Angeles, USA). The systematic difference between drug/35 mM K^+ and 35 mM K^+ alone was set as a fixed effect, with gold particle density as the dependent variable and the experiment number as a random effect.

The distribution of glutamate- and GABA-like immunoreactivities (LI) in the neuropil of CA1 was similar to that described in previous reports based on post-embedding immunogold analysis of hippocampal slices[16]. GABA-LI was concentrated in terminals establishing symmetric contacts with dendritic stems. Glutamate appeared to be more evenly distributed, consistent with the multiple roles of this amino acid in the general metabolism[7], but was clearly enriched in terminals of asymmetric synaptic contacts with spines.

In this study, a total of 1,219 nerve terminals from 16 immunocytochemical experiments were investigated. Each immunocytochemical experiment included one ultrathin section from a slice that had been incubated with drug/35 mM K^+, and one ultrathin section from the consecutive slice treated with 35 mM K^+ alone. The mean number of nerve terminals recorded in each ultrathin section was 31 (range: 5–73).

Depolarization with high K^+ concentration in the absence of drugs produced the expected decrease in the level of GABA- and glutamate-LI in the nerve terminals (Table I).

The predicted mean level of GABA-LI in terminals establishing symmetric contacts was significantly lower after exposure to 35 mM K^+ (without drug) than after exposure to 35 mM K^+ in the presence of 3α-OH-DHP (Table II, Fig. 1). The average difference was 45% (gold particle density after 3α-OH-DHP/35 mM K^+ = 100%; Table II).

With regard to the level of glutamate-LI in presumed glutamatergic terminals, there was a significant, 16% lower glutamate-LI in sections exposed to 3α-OH-DHP/35 mM K^+ compared to sections exposed to

TABLE I

Gold particle density (particles/μm^2) in terminals exposed to ACSF with normal (5 mM) or elevated (35 mM) K^+ concentration

Depolarization with 35 mM K^+ produced in all cases the expected reduction in nerve terminal contents of GABA and glutamate (GLU) expressed by immunogold labelling intensities. Experiment numbers correspond to experiment numbers in Tables II and III. *n*, number of terminals; d, density; S.D., standard deviation.

Amino acid / Exp. no.	*ACSF/5 mM K^+*			*ACSF/35 mM K^+*		
	n	*d*	*S.D.*	*n*	*d*	*S.D.*
GABA 4	29	98	23	5	32	15
5	26	80	25	18	59	34
9	14	107	34	6	25	28
11	30	78	22	19	9	12
Estimated mean (d)		90			36 *	
GLU 6	39	157	27	48	114	25
8	56	150	24	47	84	22
14	43	120	30	54	79	21
Estimated mean (d)		143			93 *	

* Significantly reduced compared to ACSF/5 mM K^+, $P < 0.0005$, (BMDP 3V, not corrected for multiple comparisons).

high K^+ alone (Table II). However, the magnitude of the reduction varied between the individual experiments.

Equivalent experiments were done with PB. Similar to 3α-OH-DHP, PB significantly counteracted the K^+-induced reduction in GABA-LI (Table III, Fig. 1). The mean level of GABA-LI after exposure to 35 mM K^+ (without drug) was only 22% of that in sections exposed to 35 mM K^+ in the presence of PB.

Compared to sections exposed to PB/35 mM K^+, sections exposed to 35 mM K^+ alone showed a significantly lower level of glutamate-LI with a predicted mean difference of 31% (Table III).

Screening for drug effects on the synaptic handling of transmitters is routinely based on biochemical analysis of synaptosome preparations. Such preparations afford a limited anatomical resolution and their heterogenous nature may easily mask even pronounced effects in an individual fibre system. This may explain why Fleischmann et al.[6], in a study of synaptosomes from rat hypothalamus and preoptic area, failed to demonstrate a statistically signifcant reduction in GABA and glutamate release after progesterone administration. The present immunocytochemical approach permits an analysis of selected fiber categories in restricted brain regions such as the CA1 subfield of hippocampus, where progesterone metabolites have been shown to have clear neurophysiological effects[21]. Incubation conditions similar to those used here give an almost linear relationship between the concentration of fixed amino acid and real density of gold particles[15], implying that any change in gold particle

TABLE II

Gold particle density (particles/μm^2) in terminals exposed to 3α-OH-DHP/35 mM K^+ or 35 mM K^+ alone

For GABA-LI, experiments 1 and 2 were done with sections from the same set of hippocampal slices, experiments 3 and 4 with sections from another set of hippocampal slices from the same animal, and experiment 5 with sections from a different animal. For glutamate (GLU)-LI, experiments 6 and 7 were done with sections from two different sets of hippocampal slices of the same animal, while experiment 8 was done with sections from a different animal. Each experiment was based on a pair of consecutive hippocampal slices, and the ultrathin sections were prosessed simultaneously in order to allow a direct comparison. Estimated mean of drug treated material was set to 100%. Other details as for Table I.

Amino acid / Exp. no.	*3α-OH-DHP/35 mM K^+*			*35 mM K^+*		
	n	*d*	*S.D.*	*n*	*d*	*S.D.*
GABA 1	29	117	26	29	68	37
2	19	62	17	21	37	25
3	32	155	40	31	101	41
4	15	73	17	5	32	15
5	36	119	39	18	59	34
Estimated mean (d)		107 (100%)			59 * (55%)	
GLU 6	46	116	30	48	114	25
7	32	148	31	51	146	29
8	50	146	28	47	84	22
Estimated mean (d)		137 (100%)			115 * (84%)	

* Significantly reduced compared to 3α-OH-DHP/35 mM K^+, $P < 0.0005$ (BMDP 3V).

TABLE III

Gold particle density (particles/μm^2) in terminals exposed to PB/35 mM K^+ or 35 mM K^+ alone

For GABA-LI, experiments 9 and 10 were done with sections from the same set of hippocampal slices, experiment 11 with sections from another set of hippocampal slices from the same animal, and experiment 12 with sections from a different animal. For glutamate (GLU)-LI, experiments 13 and 14 were done with sections from the same set of hippocampal slices, experiment 15 with sections from another set of hippocampal slices from the same animal, while experiment 16 was done with sections from a different animal. Other details as for Table I.

Amino acid / Exp. no.	*PB/35 mM K^+*			*35 mM K^+*		
	n	*d*	*S.D.*	*n*	*d*	*S.D.*
GABA 9	22	104	22	6	25	28
10	17	184	57	16	49	37
11	17	73	15	19	9.0	12
12	14	64	24	14	13	11
Estimated mean (d)		107 (100%)			23 * (22%)	
GLU 13	21	49	20	17	27	10
14	59	120	27	54	79	21
15	33	209	33	42	173	51
16	73	129	45	51	79	19
Estimated mean (d)		128 (100%)			88* (69%)	

* Significantly reduced compared to 3α-OH-DHP/35 mM K^+, $P < 0.0005$ (BMDP 3V).

density should signal a corresponding change in the concentration of the fixed amino acid.

The main finding in our study was that 3α-OH-DHP reduced the K^+-induced depletion of GABA and, to a lesser extent, that of glutamate, from nerve terminals in the CA1 field of the hippocampus. Similar results were obtained with PB. The most likely explanation for our finding is that 3α-OH-DHP, like PB, exerts an inhibitory action on GABA and glutamate release. We have previously shown in the same type of preparation that the effect of K^+ depolarization is reduced under Ca^{2+}-free conditions with high $[Mg^{2+}]$[16] suggesting that we are dealing with activation of the synaptic release process. An effect of 3α-OH-DHP on transmitter re-uptake cannot be ruled out, although barbiturates do not seem to affect GABA re-uptake in the hippocampus[9]. Another possibility, although remote, is that the nerve terminal changes are secondary to drug effects on the parent cell bodies or dendrites.

The findings concerning the effect of PB are in accordance with several other studies demonstrating that barbiturates reduce the presynaptic release of several neurotransmitters, including GABA and glutamate[4,11,24], which is probably related to inhibition of presynaptic calcium influx[18]. The concentration that inhibits synaptosomal calcium uptake is within the therapeutic plasma concentration range[18]. The presynaptic effects of progesterone may be mediated through a similar mechanism. This could be in agreement with the finding of Nikezic et al.[14] that progesterone reduces calcium uptake in synaptosomes from ovariectomized rats. Single injections of 2 mg progesterone

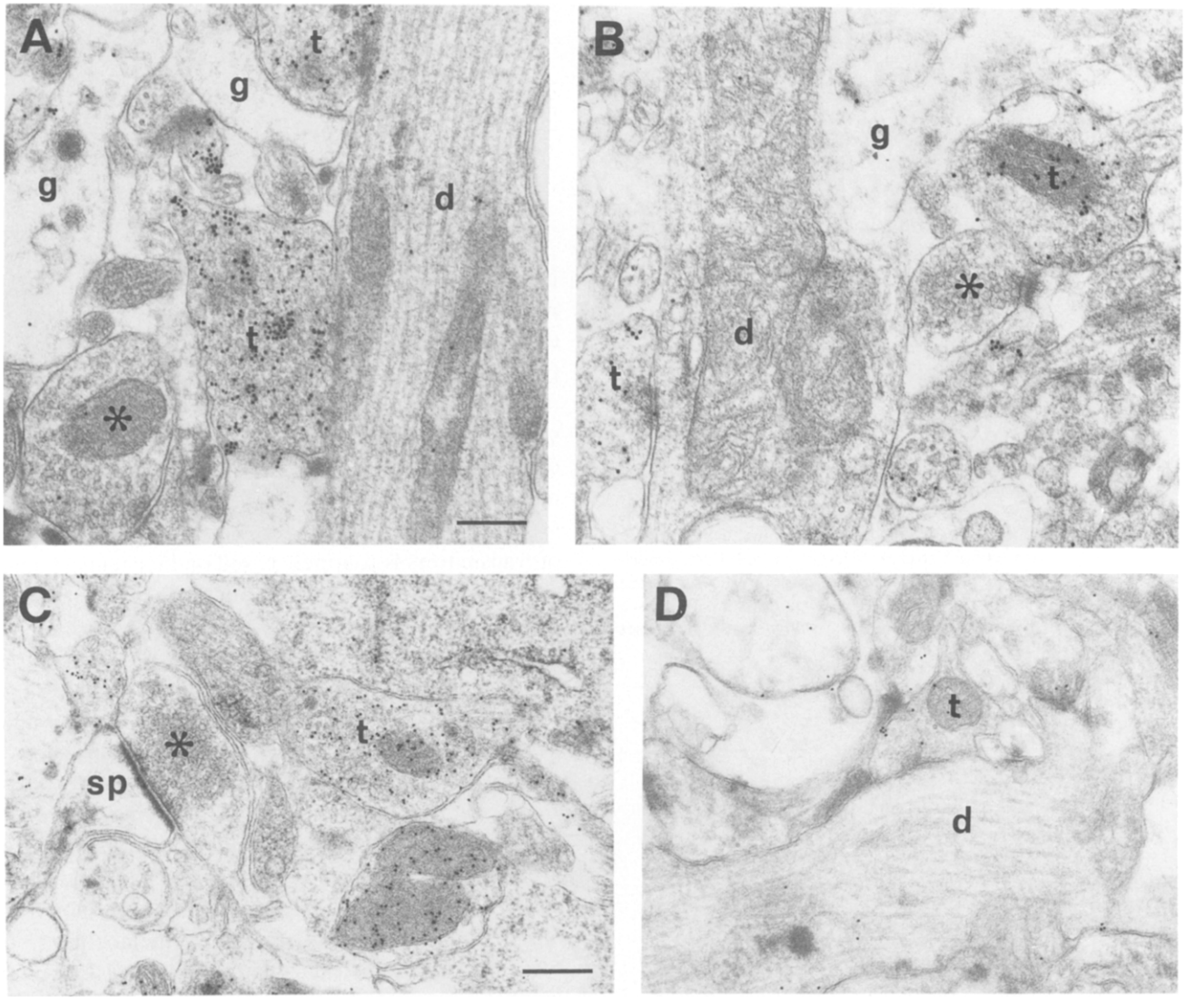

Fig. 1. A,B: GABA-LI in sections exposed to 3α-OH-DHP + 35 mM K^+ (A) or 35 mM K^+ alone (B). C,D: GABA-LI in sections exposed to PB + 35 K^+ (C) or 35 mM K^+ (D). Note the lower gold particle densities in terminals exposed to 35 mM K^+ alone, than in terminals exposed to 3α-OH-DHP with 35 mM or PB with 35 mM K^+. t, terminal; d, dendrite; g, glia; *, terminal without immuno-labelling. Bar = (A,B) 0.2 μm, (C,D) 0.3 μm.

given 24 h prior to decapitation caused a marked decrease in calcium uptake in all regions studied. The effect was especially pronounced in the hippocampus.

The present study represents one of the first reports pointing to possible presynaptic effects of progesterone metabolites. When taken together with previous data in the literature[1,6,8,10,12,14,21,22], our findings suggest that 3α-OH-DHP exerts barbiturate-like effects on GABA and glutamate release in addition to the effect on the $GABA_A$ receptor. However, conclusions as to the exact presynaptic site of action of the steroid metabolite can not be decided from our experiments, and may well differ from that of the barbiturates, which is the case post-synaptically[1,23]. Possible effects on transmitter release should be taken into account when considering the anticonvulsant properties of progesterone metabolites.

We are grateful to Dr. Morten Eriksen for his expert statistical assistance. The study was supported by The Norwegian Council for Science and the Humanities.

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