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Raised [^{3}H]-5-HT Release and $^{45}Ca^{2+}$ Uptake in Diazepam Withdrawal: Inhibition by Baclofen

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ANDREWS, N., A. ZHARKOVSKY AND S. E. FILE. *Raised [^{3}H]-5-HT release and $^{45}Ca^{2+}$ uptake in diazepam withdrawal: Inhibition by baclofen.* PHARMACOL BIOCHEM BEHAV **41**(4) 695–699, 1992.—The effect of diazepam withdrawal (2 mg/kg/day) on release of [^{3}H]-5-hydroxytryptamine(5-HT) and [^{14}C]-GABA from rat cortical and hippocampal slices was studied. No changes in [^{14}C]-GABA release (basal, K^+-evoked, uptake) from slices of either region were observed. Similarly, all parameters of [^{3}H]-5-HT release were unchanged in cortical slices. However, during diazepam withdrawal, depolarised [^{3}H]-5-HT release from hippocampal slices was raised with no changes in basal release or uptake into the slices being found. This increase could be prevented by in vivo administration of 1 mg/kg baclofen—this dose having no significant effect on [^{3}H]-5-HT release from hippocampal slices of control rats. To further investigate this effect, $^{45}Ca^{2+}$ uptake into hippocampal synaptosomes was examined and found to be increased during withdrawal. This was blocked by in vitro addition of 10 μM (−)baclofen, which had no effect on $^{45}Ca^{2+}$ uptake in controls. Inhibition of $^{45}Ca^{2+}$ uptake by (−)baclofen was also enhanced in nonwithdrawn diazepam-treated rats, but not in rats treated acutely with diazepam. The results from both studies indicate that chronic diazepam treatment increases neuronal sensitivity to baclofen. These results are discussed with reference to the anxiogenic state during diazepam withdrawal and a recent report of reversal of this behaviour by baclofen.

Diazepam withdrawal [^{3}H]-5-HT release Calcium uptake Baclofen

ANIMALS withdrawn from chronic benzodiazepine treatment show behavioural changes in animal tests indicating increased anxiety (6). These anxiogenic responses can be reversed by both agonists (diazepam) (8) and antagonists (flumazenil) (1) acting at the benzodiazepine receptor. The increased anxiety can also be reversed by low doses of the $GABA_B$ agonist, baclofen, and of the 5-HT_{1A} receptor partial agonist, buspirone (7,8).

We have previously found changes in [^{14}C]-GABA and [^{3}H]-5-hydroxytryptamine(5-HT) release from hippocampal and cortical slices taken from rats withdrawn from 3–4 weeks of diazepam (4 mg/kg/day) treatment (10). Of the changes previously reported, the increased [^{3}H]-5-HT release from the hippocampus of rats withdrawn from diazepam seemed the one most likely to mediate the anxiogenic response detected in animal tests. If the increase in [^{3}H]-5-HT release from terminal regions in the hippocampus mediated the anxiogenic response during benzodiazepine withdrawal, then reversals of this behaviour (seen in the social interaction and elevated plus maze tests of anxiety) may be effected by baclofen and buspirone (7,8) acting at presynaptic $GABA_B$ and 5-HT_{1A} receptors, respectively, in the dorsal raphe nucleus (DRN).

The purpose of Experiment 1 was to investigate changes in [^{3}H]-5-HT and [^{14}C]-GABA release from cortical and hippocampal slices taken from rats withdrawn from 21 days of diazepam (2 mg/kg/day). This dose was selected to extend our earlier release studies (we previously used 4 mg/kg diazepam) and to equate with the doses used in our behavioural studies. We also investigated the effects of acute in vivo administration of (±) baclofen (1 mg/kg) on the neurotransmitter release during withdrawal from diazepam. This dose of baclofen was selected as it had previously reversed the anxiogenic withdrawal response detected in animal tests.

The results of Experiment 1 showed increased hippocampal, but not cortical, [^{3}H]-5-HT release during diazepam withdrawal and an enhanced inhibitory action of baclofen. In Experiment 2, we therefore examined whether these changes could be due to changes in calcium uptake since presynaptic $GABA_B$ receptors are coupled to calcium channels (2) and thus control neurotransmitter release.

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METHOD

Animals

Male, hooded Lister rats (Olac, Bicester) weighing 200 g at the start of treatment were housed in groups of four in a room with lights on from 0600–1800 h. Food and water were freely available.

Drug Treatment

Rats were randomly allocated to the control (vehicle) or diazepam (2 mg/kg/day) chronic treatment groups. Animals received daily IP injections for 21 days in Experiment 1 and for 3–4 wk in Experiment 2. Diazepam (Roche Products Ltd.) was suspended in a water/Tween-20 vehicle and injected in a volume of 2 ml/kg body weight. Animals in the control treatment received equal volume injections of the water/Tween vehicle.

In Experiment 1, rats from both chronic treatments were randomly allocated to the baclofen or vehicle acute injection groups. (±)-Baclofen (Sigma) was dissolved in water and injected IP in a volume of 2 ml/kg 30 min before sacrifice and 24 h after the last of the chronic treatments. Those allocated to the vehicle acute injection received an equal volume injection of distilled water 30 min before sacrifice and 24 h after the last of the chronic treatments.

In Experiment 2, 24 h after the last chronic injection rats from both chronic treatments (vehicle or diazepam 2 mg/kg/day) were randomly allocated to receive diazepam (2 mg/kg, IP) or vehicle 30 min prior to sacrifice. There were thus four experimental groups: 1) control (chronic vehicle and acute vehicle); 2) acute diazepam (chronic vehicle and acute diazepam); 3) chronic diazepam (chronic diazepam + acute diazepam); 4) diazepam withdrawal (chronic diazepam + acute vehicle). Half the synaptosomes of a given group were incubated with, and half without, (−)baclofen.

Chemicals

4-Amino-*n*-[U-^{14}C]-butyric acid (216 m Ci/mmol) and 5-[1,2 − ^{3}H (*N*)]-hydroxytryptamine creatinine sulphate (28.4 Ci/mmol) were obtained from Amersham International. The Krebs bicarbonate buffer contained (mM): NaCl 118, KCL 4.8, $CaCl_2$ 2.4, Mg SO4 1.2, $NaHCO_3$25, KH_2PO_4 1.2, and glucose 9.5. The Krebs was continuously gassed with 95% O_2–5% CO_2.

(−)Baclofen (CIBA-GEIGY) was dissolved in distilled water to a concentration of 10 mM and then further diluted with incubation buffer on the day of assay. $^{45}CaCl_2$ (32 mCi/mg) was purchased from New England Nuclear. All other chemicals were obtained through BDH Ltd. unless stated otherwise.

Measurement of Release

The brain was removed and frontal cortex and hippocampus dissected out. Slices of both brain regions were cut using a MacIlwain tissue chopper set to cut 0.2-mm thick slices. The tissues (five slices equating to approx. 10 mg wet wt) were incubated separately in 5 ml Krebs bicarbonate buffer containing amino-oxyacetic acid, 50 μM; pargyline, 50 μM; ascorbic acid, 100 μM; and EDTA, 35 μM; (all obtained through SIGMA) at 37°C for 10 min. [^{3}H]-5-HT (0.035 μM) and [^{14}C]-GABA (0.23 μM) were added to the medium and the slices were incubated for 30 min. Slices were then placed in a chamber of 1 ml volume between two nylon grids and washed with oxygenated Krebs buffer for 15 min to obtain a steady resting rate of release. Following this washout period, perfusate was collected every 2 min at a rate of 1 ml/min. Slices were stimulated for a period of 2 min during collection of the seventh fraction with 30 mM KCl. The amounts of radiolabelled 5-HT and GABA were assessed by liquid scintillation counting [Ultima Gold (Packard) scintillation fluid was used as the counting medium] and expressed as the fractional rate coefficient (FRC). The amount of evoked release was taken as the sum of the FRC's after stimulation that were greater than the baseline FRC−taken as the mean of fractions 5 and 6. Uptake of the neurotransmitters into the slices was assessed as total dpm collected during the collection period plus the number of counts present in the tissue slices at the end of the collection period.

Preparation of Synaptosomes for $^{45}Ca^{2+}$ Uptake

Animals were killed by decapitation, brains were rapidly removed, and hippocampus dissected out on a precooled Petri

TABLE 1

MEAN (±SEM) BASAL AND K^+-STIMULATED RELEASE AND UPTAKE OF [^{14}C]-GABA FROM HIPPOCAMPAL AND CORTICAL SLICES AND [^{3}H]-5-HT FROM CORTICAL SLICES TAKEN FROM RATS TREATED FOR 21 DAYS WITH VEHICLE (CONTROL) OR WITHDRAWN FROM DIAZEPAM (2 mg/kg/day)

	Control		Diazepam Withdrawal	
	Vehicle	Baclofen	Vehicle	Baclofen
[^{14}C]-GABA Hippocampus				
Basal release	0.30 ± 0.04	0.31 ± 0.04	0.32 ± 0.05	0.34 ± 0.03
K^+stimulated	13.8 ± 2.6	16.5 ± 1.9	15.7 ± 2.3	13.9 ± 2.9
Uptake	77222 ± 7324	65834 ± 2446	72683 ± 5179	61822 ± 11493
[^{14}C]-GABA Cortex				
Basal release	0.24 ± 0.04	0.27 ± 0.05	0.29 ± 0.04	0.29 ± 0.06
K^+stimulated	10.6 ± 1.4	12.0 ± 1.1	12.1 ± 1.9	15.1 ± 2.2
Uptake	85851 ± 8941	84296 ± 10308	74343 ± 6677	58167 ± 7312
[^{3}H]-5-HT Cortex				
Basal release	0.86 ± 0.10	0.74 ± 0.07	0.90 ± 0.05	0.91 ± 0.12
K^+stimulated	7.7 ± 1.9	8.1 ± 1.6	8.8 ± 1.8	10.3 ± 1.9
Uptake	372769 ± 37908	444021 ± 39551	347182 ± 26573	288204 ± 37020

Rats were killed 24 h after the last chronic injection and 30 min after IP acute injection with vehicle or baclofen (1 mg/kg).

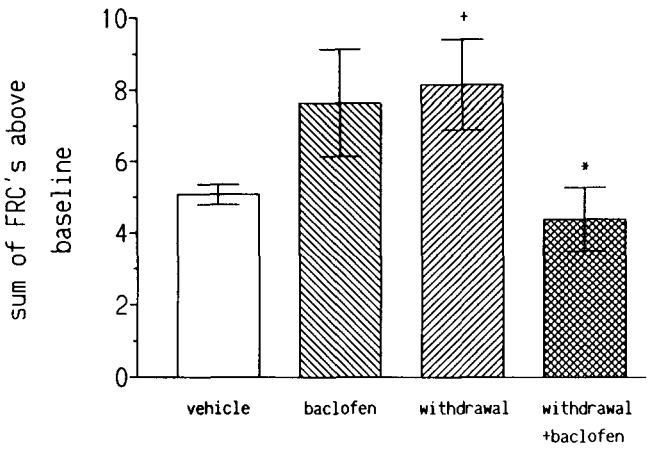

FIG. 1. Mean (±SEM) K^+-stimulated [^{3}H]-5-HT release from hippocampal slices taken from rats treated for 21 days with vehicle (control) or withdrawn from diazepam (2 mg/kg/day for 21 days). Rats were killed 30 min after IP injection with vehicle or with baclofen (1 mg/kg). $^*p < 0.05$ compared with untreated withdrawal group; $^+p < 0.05$ compared with control group.

dish. Tissues were homogenized in 10 vol. ice-cold homogenization buffer [0.32 M sucrose (Sigma) in 0.004 mM Tris-base buffer (Sigma); pH adjusted to 7.4 with 1 N HCl] using a 2-ml volume glass homogenizer with Teflon pestle (10 strokes up and down). The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant recentrifuged at 20,000 *g* for 20 min at 0–4°C. The resulting pellet containing the P_2 crude synaptosomal fraction was resuspended in homogenization buffer to give a protein concentration of approximately 1 mg/ml (range = 0.9–1.5 mg/ml).

$^{45}Ca^{2+}$ Uptake Assay

All assays were performed in duplicate in a volume of 1 ml. First, 0.1 ml synaptosomal preparation was added to the assay tubes containing 0.7 ml incubation buffer (136 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 11 mM glucose, and 20 mM Tris-base; pH adjusted to 7.4 with 1 N HCl) and kept on ice for 10 min. At the end of the preincubation period, 0.1 ml incubation buffer with or without baclofen (10 μM) was added to the tubes containing synaptosomes from each of the four experimental groups and allowed to equilibrate for a further 10 min at 36°C in a shaking water bath. The uptake was initiated by adding 0.1 ml $^{45}CaCl_2$ solution (100 μM final concentration; 0.75 μCi per assay tube) containing either 20 mM KCl (final concentration) for depolarizing the membranes or equivalent concentration of NaCl for basal uptake measurement. The incubation was then continued for 60 s and terminated by the rapid addition of ice-cold stopping solution (145 mM KCl, 2.5 mM $CaCl_2$, and 20 mM Tris-base; pH adjusted to 7.4 with 1 N HCl) to the incubation tubes and immediate filtration through Whatman GF/B filters presoaked with 0.0005% polyethyleneimine solution. Filters were additionally washed four times with 4 ml stopping solution and placed into plastic vials along with 8 ml Emulsifier Safe (Packard). Samples were counted 18 h later using a 1214 RackBeta (LKB Wallac) scintillation counter. Synaptosomal protein concentration was assayed by the method of Lowry et al. $^{45}Ca^{2+}$ uptake was expressed in nmol of Ca^{2+} taken up per mg of synaptosomal protein and net uptake was calculated by subtracting the basal (nondepolarised values; 20 mM NaCl) from the depolarized (20 mM KCl) values.

Statistics

In Experiment 1, the data were analysed with two-way analyses of variance (ANOVA) with the chronic treatment (control or diazepam) as one factor and the acute injection (vehicle or baclofen) as the second.

In Experiment 2, the data were analysed by a three-way split-plot ANOVA with the same two independent factors and the in vitro treatment (control or baclofen) as the related measure. Posthoc Duncan's test was utilized where necessary.

RESULTS

[^{14}C]-GABA Release

There were no significant changes in basal or K^+-stimulated [^{14}C]-GABA release or in uptake from the hippocampus when control-treated animals were compared with the diazepam-withdrawn group. In vivo administration of baclofen (1 mg/kg) was also without significant effects in either of the pretreatment groups (see Table 1). There were also no significant changes in [^{14}C]-GABA release or uptake from cortical slices (see Table 1).

[^{3}H]-5-HT Release

There were no significant changes in basal or K^+-stimulated [^{3}H]-5-HT release from cortical slices of diazepam-withdrawn rats compared with controls, nor were there any significant effects of in vivo baclofen administration (see Table 1).

However, there was a significant increase in K^+-stimulated [^{3}H]-5-HT release from hippocampal slices of diazepam-withdrawn rats compared with controls (see Fig. 1). In addition, the effects of baclofen (1 mg/kg, IP) were different in the two chronic pretreatment groups [baclofen × pretreatment interaction, $F(1,18) = 7.41$, $p < 0.005$], with a nonsignificant enhanced release in the control-treated group and a significant inhibition of release in the diazepam-withdrawn group (see Fig. 1). There were no changes in basal release or uptake of [^{3}H]-5-HT in the hippocampus (data not shown).

^{45}Calcium Uptake

There were no significant effects of diazepam or baclofen on basal calcium uptake (see Table 2).

Baclofen significantly reduced net calcium uptake, $F(1,28) = 12.4$, $p < 0.005$, and this effect was much more marked in animals that had chronic pretreatment with diazepam [chronic treatment × baclofen interaction, $F(1,28) = 5.9$, $p < 0.05$] (see Fig. 2). The effect of baclofen was not altered by acute in vivo administration of diazepam prior to sacrifice (see Fig. 2).

It can also be seen from Fig. 2 that although the acute administration of diazepam did not change baclofen's effects in all groups diazepam reduced calcium uptake, $F(1,28) = 5.7$, $p < 0.05$. Although this effect appears more marked in the groups chronically pretreated with diazepam, this did not reach significance [chronic treatment × acute diazepam interaction, $F(1,28) = 1.6$].

Withdrawal from 28 days of diazepam treatment resulted in a significant elevation of calcium flux compared with the vehicle-treated group (Duncan's test, $p < 0.05$) (see Fig. 2).

TABLE 2

MEAN (±SEM) BASAL $^{45}Ca^{2+}$ UPTAKE INTO SYNAPTOSOMES OF RATS INJECTED IP FOR 3-4 WEEKS WITH EITHER VEHICLE OR DIAZEPAM (2 mg/kg/day)

	Chronic Vehicle	Acute Diazepam	Chronic Diazepam	Diazepam Withdrawal
Baclofen absent	1.34 ± 0.19	1.24 ± 0.15	1.21 ± 0.17	1.30 ± 0.14
Baclofen present	1.20 ± 0.12	1.22 ± 0.14	1.14 ± 0.19	1.15 ± 0.11

Rats were killed 30 min after the last IP injection. Synaptosomes from the same animal were incubated in the presence and absence of 10 μM(−)baclofen prior to addition of $^{45}Ca^{2+}$.

DISCUSSION

Our finding of increased [^{3}H]-5-HT release from hippocampal slices during diazepam withdrawal supports the results from our previous experiments (10) using a higher dose of diazepam (4 mg/kg, IP), but with this lower dose no other significant changes were detected. This suggests that the decreased [^{3}H]-5-HT release in the cortex and the increased [^{14}C]-GABA release in the hippocampus previously found upon withdrawal from the higher dose of diazepam of our previous study do not mediate the anxiogenic withdrawal response since anxiogenic behaviour is clearly detectable on withdrawal from 21 days of treatment with 2 mg/kg diazepam (7,8).

The changes we observed in calcium flux in the hippocampus during withdrawal from chronic benzodiazepine treatment appear to be, at least to some extent, regionally specific. No change in basal or potassium-stimulated fast- or slow-phase calcium uptake were observed in cortical synaptosomes from rats withdrawn from chronic diazepam treatment (11). This would be in accordance with our finding of increased [^{3}H]-5-HT release from hippocampal but not cortical slices.

The marked change in the effects of in vivo baclofen (1 mg/kg) on hippocampal [^{3}H]-5-HT release suggest that with chronic diazepam treatment there is a change in sensitivity to baclofen. Chronic treatment with diazepam also induced a marked increase in sensitivity to the inhibitory effects of baclofen on calcium uptake, which is likely to underlie the change in neurotransmitter release. A similar change in sensitivity to baclofen has been observed at the behavioural level: Doses of baclofen ineffective in control-treated rats significantly reversed the anxiogenic effects detected during diazepam withdrawal (8).

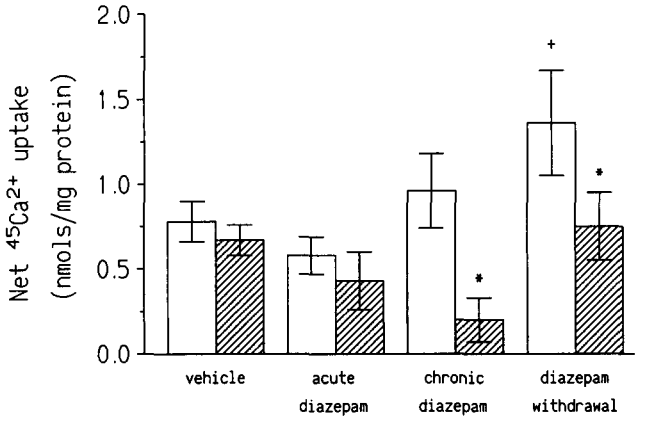

FIG. 2. Mean (±SEM) changes in net (K^+-stimulated-basal) ^{45}Ca uptake of rats treated for 3-4 weeks with vehicle or with diazepam (2 mg/kg/day). Twenty four h after the last chronic injection and 30 min prior to sacrifice, animals received either vehicle (vehicle and diazepam withdrawal groups) or 2 mg/kg diazepam (acute and chronic diazepam groups). The hatched columns show the effects of in vitro addition of (−)baclofen (10 μM) to each synaptosomal preparation. *$p < 0.05$ compared with absence of baclofen; $^+p < 0.05$ compared with vehicle control group.

The mechanism mediating the alteration in sensitivity to baclofen inhibition of calcium uptake in the hippocampus, during benzodiazepine withdrawal, is unknown. One possibility is an increased number of functional presynaptic $GABA_B$ receptors on terminal regions of serotonergic neurons projecting to the hippocampus. $GABA_B$ receptors have been shown to upregulate following chronic treatment with other psychotropic drugs including antidepressants (14), lithium, and carbamazepine (13). Upregulation of $GABA_B$ receptors, if they were of the type described by Gray and Green (9), that is, heteroreceptors regulating neuronal 5-HT release, would provide an explanation for the observed enhanced sensitivity to baclofen (seen in both Experiments 1 and 2) during withdrawal. Upregulation of $GABA_B$ receptors would almost certainly not be confined to the hippocampus, but would also occur in other areas important for serotonergic transmission, for example, the raphe nuclei, particularly the DRN. Bowery et al. (3) demonstrated the presence of $GABA_B$ binding sites in the DRN. The DRN contains the cell bodies of serotonergic neurons, and agonist occupation of $GABA_B$ receptors on the cell bodies would lead to an eventual decrease in release of 5-HT from terminals in the hippocampus. We have not yet looked at regional changes in $GABA_B$ binding during benzodiazepine withdrawal, but autoradiography (rather than homogenate binding) would be a useful way to investigate this. A change in coupling of the receptors to their regulatory G proteins may also be envisaged as another way in which sensitivity to baclofen could be increased during withdrawal.

Another possible mechanism that could account for the change in sensitivity to baclofen after chronic treatment with diazepam is an upregulation of voltage-dependent calcium channels. Although Dolin et al. (5) found no changes in [^{3}H]-nitrendipine binding in brains of mice withdrawn from chronic benzodiazepine administration, there is evidence from cultured bovine adrenal chromaffin cells that upregulation of dihydropyridine-sensitive calcium channels occurs after chronic exposure to benzodiazepines (4).

In conclusion, we have found evidence of increased hippocampal calcium flux during benzodiazepine withdrawal, which could underlie the increased hippocampal [^{3}H]-5-HT release and the supersensitivity to the inhibitory effects of baclofen. This action could underlie the behavioural reversals by baclofen of the anxiogenic responses detected during diazepam withdrawal.

ACKNOWLEDGEMENTS

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