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Effects of caffeine on NMDA-evoked $^{45}Ca^{2+}$ release in the rat dentate gyrus *in vivo*

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Abstract. Caffeine in 10^{-2} M concentration *per se* activates ryanodine receptors (RyR) *in vitro*, thereby increasing the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). In general opinion, caffeine applied *in vivo* in much lower doses does not affect $[Ca^{2+}]_i$ in neurones. However, it was recently demonstrated that caffeine in low concentrations *in vitro* potentiates evoked Ca^{2+} release in neurones *via* RyR. Microdialysis of the rat dentate gyrus (DG), combined with measurement of $^{45}Ca^{2+}$ efflux, has been used in our laboratory to study *in vivo* NMDA-evoked calcium induced calcium release (CICR) *via* RyR. The aim of the present microdialysis study was to investigate *in vivo* effects of caffeine, applied systemically in a pharmacologically-relevant dose, and locally in the dialysis medium in very high concentration, on the NMDA-evoked CICR in DG neurones. To ensure steady brain concentration of caffeine, its systemic (i.p.) administration in a dose of 40 mg/kg was followed by a continuous i.p. infusion of 80 μg/kg/min and application of 0.4 mM caffeine in the dialysis medium. The results demonstrated that in the rat DG, local administration of 50 mM caffeine significantly stimulates a spontaneous $^{45}Ca^{2+}$ efflux and its release induced by 5 mM NMDA. However, systemic administration of caffeine had no effect on spontaneous and NMDA-induced $^{45}Ca^{2+}$ release in the rat DG, which supports the view that caffeine, applied *in vivo,* even in high doses, does not influence CICR in brain neurones.

Key words: caffeine, CICR, dentate gyrus, microdialysis, rat, ryanodine receptors

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INTRODUCTION

Mobilisation of intracellular Ca^{2+} stores, localised in endoplasmic reticulum (ER), through receptors sensitive to ryanodine (RyR) or IP_3 (IP_3R), plays a key role in generation of the calcium signal (for reviews see Ehrlich et al. 1994, Ogawa 1994, Sitsapesan et al. 1995, Simpson et al. 1995, Mody and MacDonald 1995, Kostyuk and Verkhratsky 1995). In the hippocampal formation of the rat brain, RyR are mainly expressed in dentate granule neurones, while IP_3R are almost exclusively present in CA1 neurones of the hippocampus (Worley et al. 1989, Nio et al. 1991, Sharp et al. 1993). Recently, using the microdialysis technique in rat dentate gyrus (DG) *in vivo*, combined with radio-labelling of endogenous Ca^{2+} and measurement of $^{45}Ca^{2+}$ efflux, we demonstrated the NMDA - evoked $^{45}Ca^{2+}$ release to dialysate. Pharmacological characteristics of this phenomenon correspond to Ca^{2+} - induced Ca^{2+} release (CICR) throughout RyR (Łazarewicz et al. 1998).

A constantly growing list of pharmacological tools modulating $[Ca^{2+}]_i$ *in vitro* includes caffeine, which activates the most important RyR isoforms (Ehrlich et al. 1994, Simpson et al. 1995) but has been recognised as an inhibitor of IP_3R (Parker and Ivorra 1991, Brown et al. 1992, Ehrlich et al. 1994). At least 5 mM caffeine (50 times its pharmacological concentration) is necessary to produce Ca^{2+}-independent activation of RyR and to inhibit IP_3R *in vitro* (Sitsapesan and Williams 1990, Nehlig et al. 1992, Ehrlich et al. 1994). Therefore, cellular mechanisms of caffeine action in the central nervous system *in vivo* have been explained by inhibition of phosphodiesterases, antagonism of adenosine receptors, and binding to benzodiazepine receptors, whereas *in vivo* caffeine effects on the intracellular mobilisation of Ca^{2+} has been generally rejected (Nehlig et al. 1992).

It was recently reported that caffeine in high micromolar concentrations increases the size of the $[Ca^{2+}]_i$ transients induced by depolarisation in snail neurones *in vitro*, *via* potentiation of Ca^{2+}-dependent activation of RyR (Orkand and Thomas 1995). It was also demonstrated that 0.5 mM to 2 mM caffeine sensitises to Ca^{2+} sheep cardiac RyR incorporated into artificial phospholipid membranes (Sitsapesan and Williams 1990, Sitsapesan et al. 1995). However, it has not been proven if caffeine applied *in vivo* in pharmacologically active doses sensitises RyR to Ca^{2+} in brain neurones. Such an effect of caffeine should result in potentiation of CICR, evoked by factors that increase Ca^{2+} influx to neurones, e.g., by NMDA receptor stimulation. Thus, our *in vivo* microdialysis model of NMDA-evoked CICR in the rat hippocampus seems to be suitable for studies of caffeine effects on RyR.

An intrinsic feature of the brain microdialysis is drainage of the substances present in the extracellular fluid in the vicinity of the dialysis probe (Benveniste et al. 1988). This applies to endogenous substances and to drugs administered systemically, penetrating the blood-brain barrier. Thus, an additional task of this study was to prevent caffeine drainage.

The aim of this work was to investigate *in vivo* influence of caffeine, administered systemically in a high pharmacologically active dose, in comparison to effects of its very high dose applied locally, on NMDA-evoked Ca^{2+}-induced $^{45}Ca^{2+}$ release in the rat DG. For this purpose, a protocol of systemic caffeine application in the microdialysis experiments has been developed, which stabilises its tissue levels and prevents drainage.

METHODS

Microdialysis of the rat hippocampal formation *in vivo*

Microdialysis experiments were carried out exactly as described previously (Łazarewicz and Rybkowski 1996, Łazarewicz et al. 1998). Adult Wistar rats weighing 250-300 g were anaesthetised with urethane (1.25 g/kg b.w., i.p.). The animals were immobilised in a rat stereotaxic frame for implantation of 1 mm coaxial microdialysis probes (CMA/11, CMA/Microdialysis AB, Stockholm, Sweden). Stereotaxic coordinates for implantation to DG were: AP -4.5 mm, LR 3.0 mm relative to bregma, and -3.5 mm from the cortex (Zilles 1985). The position of the probes was determined by macroscopic examination of the brain after each experiment. The probes were perfused with Krebs-Ringer bicarbonate (K-R) medium, pH 7.4, at a rate of 2.5 µl/min. In different stages of the experiments the dialysis medium contained $^{45}CaCl_2$, N-methyl-D-aspartic acid (NMDA), and/or caffeine, in concentrations as described below.

Measurement of $^{45}Ca^{2+}$ efflux

A previously described procedure was used to detect changes in $^{45}Ca^{2+}$ efflux (Łazarewicz and Rybkowski 1996, Łazarewicz et al. 1998). Initially the probes were perfused for 1 h with K-R medium containing $^{45}CaCl_2$

(25 μCi), to pre-label the endogenous pool of calcium. This was followed by 3 h equilibration perfusion with non-radioactive K-R medium. Then samples were collected every 5 min. Radioactivity of dialysates was measured by liquid scintillation counting using a Wallac 1409 counter (LKB, Vienna, Austria). Results were extrapolated by computer-aided semilog regression analysis and were presented as a percentage of basal $^{45}Ca^{2+}$ efflux. To normalise the influence of caffeine application on NMDA-evoked $^{45}Ca^{2+}$ release, NMDA was applied in two consecutive pulses, and the area under each peak of NMDA-evoked $^{45}Ca^{2+}$ efflux was determined and peak-to-peak ratios were calculated. Caffeine was administered after the first NMDA pulse.

Protocols of caffeine application

Intraperitoneal (i.p.) injection of a 50 mM solution of base caffeine in physiological saline in a dose of 40 mg/kg was followed by continuous i.p. infusion of the same solution of caffeine in a dose of 80 μg/kg/min and application of 400 μM caffeine to the dialysis medium. In other experiments 50 mM caffeine was applied directly to the dialysis medium, in which NaCl concentration was reduced to maintain isoosmolarity. Control experiments indicated a lack of effect of such modification in NaCl concentration in the dialysis medium on spontaneous and NMDA-evoked $^{45}Ca^{2+}$ efflux.

Measurements of caffeine concentration

Caffeine measurements were carried out according to the spectrophotometric method described by Thithapandha et al. (1972). For caffeine determination in rat plasma, the animals were anaesthetised as described for microdialysis experiments, and their femoral artery was catheterised. Blood samples were collected to heparinised beakers, centrifuged, and 0.5 - 2.0 ml aliquots of plasma, or collected samples of microdialysates, were adjusted to a volume of 2.0 ml with phosphate buffer. Caffeine was extracted with 20 ml of benzene, and 15 ml aliquots of the benzene extract were washed with 2 ml of 67 mM phosphate buffer (pH 7.4). The caffeine was recovered to the aqueous phase by shaking the organic phase with 1.0 ml of 4 N HCl. To remove traces of benzene, the acid extract was washed with 1.0 ml n-heptane before the optical density was read at 273 nm, using DU 65 Beckman spectrophotometer (Beckman, Fullerton, CA, USA).

Extracellular caffeine concentration in rat brain *in vivo* was estimated by the difference method, described by Arner and Bolinder (1991) for detection of glucose and other endogenous metabolites, and by Ståhle (1991) and Ståhle et al. (1991) for caffeine. Briefly, different concentrations of caffeine were added to the dialysis medium in animals, that received i.p. injections/infusions of caffeine. The measurement was performed when a steady-state level of caffeine concentration in plasma was reached. Differences between the caffeine concentrations added to the dialysis medium and detected in dialysates were plotted against concentrations of caffeine added in the dialysis medium. A concentration that does not change as a result of flow through the microdialysis probe, determined by linear regression method, indicates the extracellular concentration of caffeine.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). Differences between means were tested using an analysis of variance (ANOVA) followed by Dunnett's test or Student's *t*-test.

RESULTS

Caffeine concentrations in rat plasma and brain

Initial experiments of this study allowed adjustment of the protocol of systemic caffeine administration, which secures a stable caffeine concentration in the blood and brain for the whole period of microdialysis experiment, and counteracts its drainage from the tissues. Figure 1 indicates that i.p. administration of caffeine in doses of 10, 20, and 40 mg/kg, supplemented with subsequent constant i.p. infusion of 26, 40 and 80 μg/kg/min respectively, resulted in stable caffeine concentrations in blood at the levels of 140, 240 and 450 μM.

In our present study, the actual caffeine concentration in the tissue fluid, after i.p. injection of 40 mg/kg b.w., supplemented with 80 μg/kg/min infusion, was estimated during the period of steady plasma concentration at the level of 450 μM. Different caffeine concentrations were added to the dialysis medium, and changes of caffeine concentrations in dialysates were measured. As presented in Figure 2, at concentrations of caffeine in the dialysis medium <400 μM, a net increase in the caffeine content in dialysate was observed. Application of higher caffeine concentrations resulted in reduction of its level

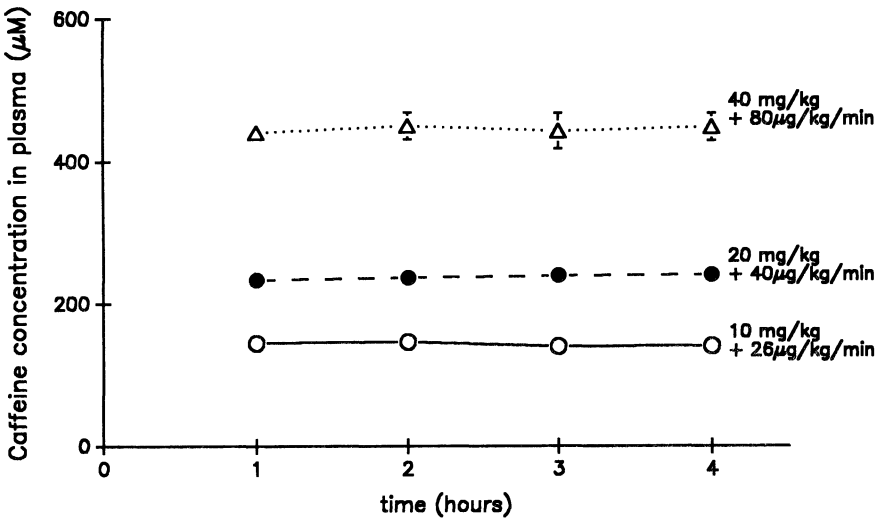

Fig. 1. Stable concentrations of caffeine in rat plasma after i.p. injection of the drug in different doses, supplemented with its continuous infusion, to compensate caffeine decay. Results are means ± SEM (n = 3 experiments).

in dialysate. A lack of changes in caffeine concentration was found at about 400 μM caffeine concentration in the dialysis medium, which indicates that this is the actual caffeine concentration in the extracellular fluid of the rat DG. Consequently, to prevent caffeine drainage, in our subsequent experiments 400 μM caffeine was added to the dialysis medium in parallel to its peripheral application.

Effects of caffeine on NMDA - evoked $^{45}Ca^{2+}$ release in the rat GD *in vivo*

As presented in Figure 3, application to the rat DG of 5 mM NMDA in the dialysis medium for 20 min leads to release of $^{45}Ca^{2+}$ to dialysate. Repeated application of the same dose of NMDA induces a similar effect, which is, however, reduced by about 1/3. Although we have noticed a considerable individual variability in the level of NMDA - evoked $^{45}Ca^{2+}$ release in the rat DG, the ratio of $^{45}Ca^{2+}$ release evoked by the second NMDA application to the first one remained constant (Table I). Therefore, to normalise the results, the effects of caffeine, applied before the second NMDA pulse, were expressed as ratios of the second to the first $^{45}Ca^{2+}$ release, and compared with a corresponding control value.

As presented in Table I, caffeine administered i.p. at a dose of 40 mg/kg, with its supplementary infusion and local application to the dialysis medium, which ensures a constant caffeine concentration in the extracellular space at the level of 400 μM, had no significant effect on NMDA - evoked $^{45}Ca^{2+}$ release in the rat DG. By contrast, application of 50 mM caffeine directly to the DG

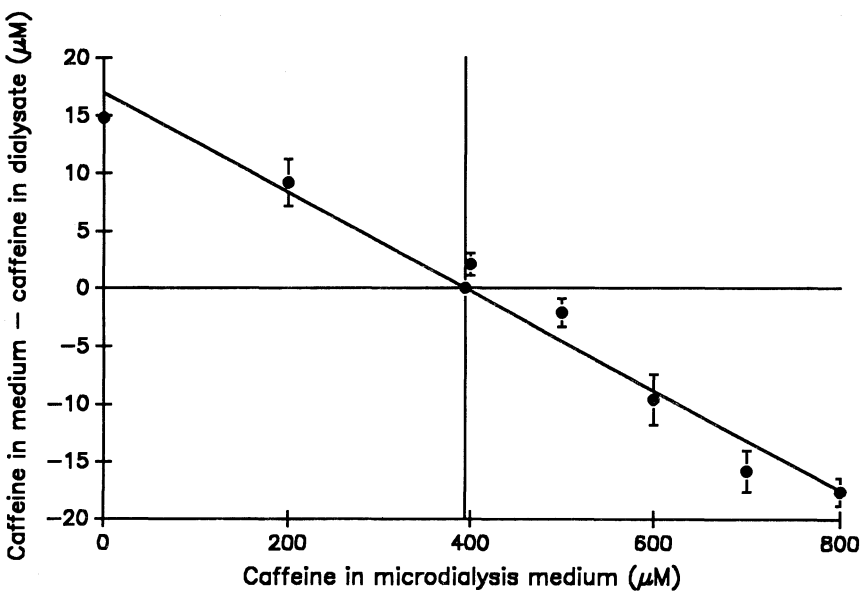

Fig. 2. Estimation of caffeine concentration in the extracellular fluid of the rat brain after i.p. injection of 40 mg/kg caffeine, followed by its infusion at the rate of 80 μg/kg/min. Caffeine in different concentrations was applied in dialysis medium, and differences between caffeine concentrations in the medium and in dialysates were detected. Caffeine concentration which does not change during microdialysis, calculated by the linear regresion (394.2 μM), equals extracellular caffeine concentration. Means ± SEM (n = 4 experiments).

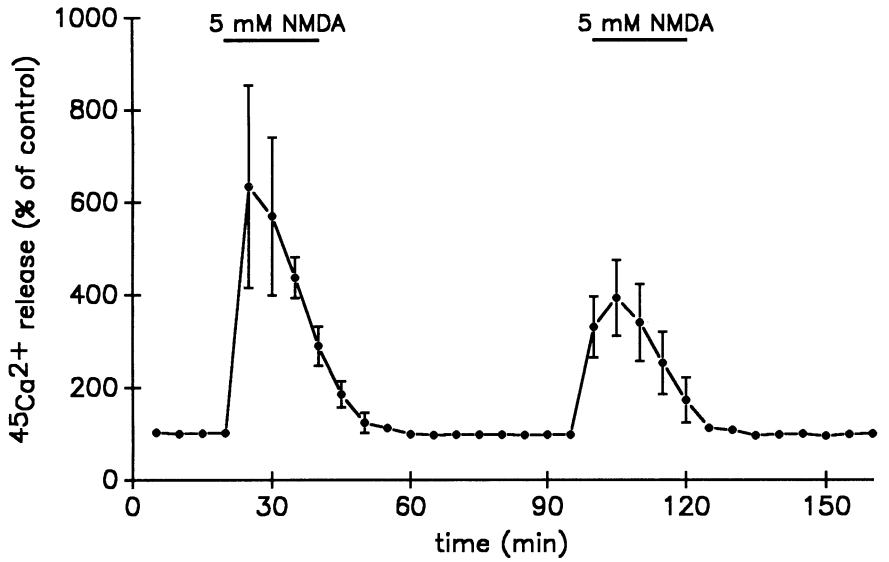

Fig. 3. Effect of repeated NMDA application in the dialysis medium on $^{45}Ca^{2+}$ efflux in dialysates of the rat gentate gyrus. After pre-labelling of the tissue with $^{45}Ca^{2+}$ *via* dialysis medium and equilibration perfusion, samples of dialysates were collected in 5-min intervals. Two consecutive pulses of 5 mM NMDA were applied in the dialysis medium as indicated by horizontal bars. Means ± SEM ($n = 4$ experiments).

via the dialysis medium resulted in a large stimulation of the release of $^{45}Ca^{2+}$ evoked by NMDA. Systemic caffeine administration had no significant effect on spontaneous $^{45}Ca^{2+}$ efflux, which was stimulated by about 70% by locally applied 50 mM caffeine (Fig. 4).

TABLE I

$^{45}Ca^{2+}$ release evoked by 5 mM NMDA in the rat dentate gyrus: modulation by caffeine

	Peak II/peak I Ratio	Change (%)
Control	0.6 ± 0.06	
Caffeine(50 mM) in dialysis medium	6.8 ± 0.66*	+ 1133
Caffeine administered systemically	0.5 ± 0.17	- 17

Peak II/peak I ratio represents a ratio of the second $^{45}Ca^{2+}$ release peak area above the control level, evoked by 5 mM NMDA under experimental conditions indicated in the table, to the first $^{45}Ca^{2+}$ release peak area evoked by 5 mM NMDA given in control dialysis medium (compare Fig. 3). Systemic (i.p.) administration of caffeine, 40 mg/kg was accompanied by i.p. infusion at a rate of 80 μg/kg/min and by application of 400 μM caffeine in dialysis medium. Data are means ± SEM values ($n = 4$-9 experiments). *Means different from the control at $P<0.05$.

DISCUSSION

In this study microdialysis, combined with detection of $^{45}Ca^{2+}$ efflux, was utilised to evaluate *in vivo* effects of caffeine on NMDA-evoked mobilisation of intracellular Ca^{2+} in the rat hippocampal formation. These results demonstrate a lack of effect of caffeine applied systemically, in a pharmacologically active dose of 40 mg/kg, on $^{45}Ca^{2+}$ release evoked by NMDA in the DG. On the other hand, 50 mM caffeine applied locally *via* the dialysis medium stimulated a spontaneous $^{45}Ca^{2+}$ efflux, and significantly potentiated NMDA-evoked $^{45}Ca^{2+}$ release in the rat DG. These results indicate that our experimental model is suitable to detect stimulatory effects of caffeine on the activity of neuronal RyR. However, utilising this model, we were not able to detect effects of caffeine applied *in vivo* in pharmacologically relevant doses on NMDA-evoked mobilisation of $^{45}Ca^{2+}$ *via* RyR.

In the experiments involving systemic caffeine administration, particular attention was paid to measure the actual extracellular caffeine concentration in the brain and to ensure its constant level during the whole experimental procedure, preventing its elimination due to metabolism, diffusion and drainage to the dialysate. Caffeine very rapidly reaches its maximal concentration in blood after i.p. injection (Teschemacher et al. 1968). Caffeine easily penetrates the blood-brain barrier (Kaplan et al. 1989), and its brain concentration rapidly equilibrates with the plasma level (Ståhle 1991, Ståhle et al. 1991). Caffeine concentration in plasma declines with $t_{1/2} = 4.2$ h (Thithapandha et al. 1972). In our study a caffeine decay was compensated by its continuous infusion.

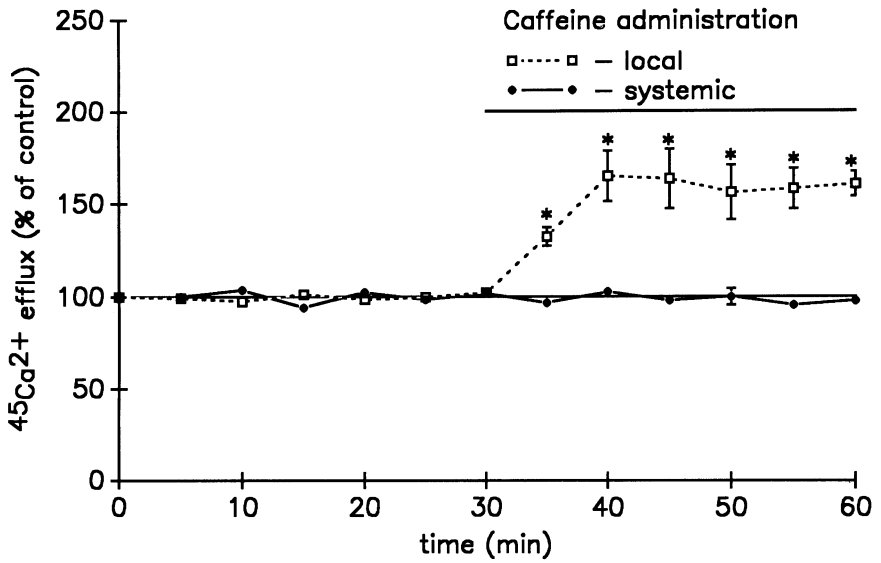

Fig. 4. Effects of systemic and local administration of caffeine on spontaneous $^{45}Ca^{2+}$ efflux in the rat dentate gyrus *in vivo*. Caffeine was added in 50 mM concentration to dialysis medium, or injected i.p. in a dose of 40 mg/kg, followed by i.p. infusion at a rate of 80 μg/kg/min, and by a constant application of 400 μM caffeine in dialysis medium. Period of caffeine application indicated by a horizontal bar. Means ± SEM ($n = 4$ experiments). *Means different from the control at $P<0.05$.

In our experiments, caffeine concentration in the extracellular fluid in brain was close to 400 μM. This level of caffeine is certainly too low to activate RyR in a Ca^{2+}-independent manner. However, it corresponds to caffeine concentrations used in experiments *in vitro*, which demonstrated potentiation by caffeine of RyR activity *via* Ca^{2+}-dependent mechanisms (Sitsapesan and Williams 1990, Sitsapesan et al. 1995, Orkand and Thomas 1995). In some experiments very high 50 mM caffeine concentrations were administered *via* the dialysis medium. This was due to a diffusion barrier across a semi-permeable dialysis membrane. In microdialysis experiments, active substances are usually applied in concentrations 5-10 times higher than effective concentrations of these substances *in vitro*. Thus, we estimate that 50 mM caffeine administered in the dialysis medium corresponds to an effective concentration of caffeine in the brain tissue in the range of 5-10 mM. This concentration is adequate to reveal a calcium-independent mechanism of RyR activation by caffeine (Sitsapesan and Williams 1990, Ehrlich et al. 1994).

The main goal of this study was to test if caffeine administered *in vivo*, and present in the mammalian brain in pharmacologically relevant micromolar concentrations, may potentiate Ca^{2+}-dependent activation of RyR (Sitsapesan and Williams 1990, Orkand and Thomas 1995, Sitsapesan et al. 1995). For this reason our experimental model of NMDA-evoked CICR was utilised. Our recent studies (Łazarewicz et al. 1998) revealed a 77.8% inhibition of NMDA-evoked $^{45}Ca^{2+}$ release in the rat DG by 100 μM dantrolene, and 43.7% inhibition by 250 μM ryanodine. In the same study 50 μM ryanodine stimulated this effect nearly 20-fold. This is consistent with well known pharmacological characteristics of RyR (for review see Ehrlich et al. 1994), and may serve as evidence for the key role of CICR *via* RyR in the NMDA-evoked $^{45}Ca^{2+}$ release in the rat DG. It is known from a number of autoradiographic and immunocytochemical studies, confirmed by *in situ* hybridisation of mRNA for RyR, that the dentate granule neurones are abounding with the mammalian cardiac type RyR (RyR_2) (Lai et al. 1992, Varma et al. 1992, Sharp et al. 1993, Furuichi et al. 1994). This RyR isoform is sensitive to modulation by caffeine, whereas sensitivity of the so-called brain type RyR (RyR_3) has been debated (Giannini et al. 1992, Murayama and Ogawa 1996, 1997, Chen et al. 1997). On the other hand, IP_3R are selectively located in the CA1 region of the hippocampus (Worley et al. 1989). Thus, our *in vivo* model of NMDA-evoked CICR utilising microdialysis of the rat DG and measurement of $^{45}Ca^{2+}$ efflux enables selective investigation of *in vivo* effects of caffeine on $^{45}Ca^{2+}$ release *via* RyR_2.

Positive results of the control experiments, i.e., the expected stimulation of spontaneous $^{45}Ca^{2+}$ efflux and NMDA-evoked release in the rat DG by locally applied 50 mM caffeine, confirmed the utility of our microdialysis model in studies of *in vivo* effects of caffeine on intracellular Ca^{2+} mobilisation. This effect is consistent with classical activation of RyR by caffeine in millimolar concentration, well known from numerous *in vitro* studies reviewed by Ehrlich et al. (1994) and Kostyuk and Verkhratsky (1995). Our preliminary experiments demonstrated also a potent inhibition of the NMDA-evoked $^{45}Ca^{2+}$ release in the hippocampus CA1 *in vivo*

in the presence of 50 mM caffeine in the dialysis medium (Alaraj and Łazarewicz, unpublished data). This effect corresponds to caffeine-evoked inhibition of IP_3R, found *in vitro* by Parker and Ivorra (1991), Brown et al. (1992) and Ehrlich et al. (1994).

These *in vivo* effects of high concentrations of caffeine may be most probably attributed to a direct interference of caffeine with RyR and IP_3R, rather than to contribution of an indirect modulation by caffeine of the activity of NMDA receptors. Inhibition or potentiation by caffeine of the NMDA receptor activity could influence the NMDA-evoked changes in $^{45}Ca^{2+}$ efflux. Only a long-term treatment of animals with caffeine was shown to decrease their susceptibility to NMDA-induced seizures (Georgiev et al. 1993), whereas acute treatment with methylxantines causes an increased susceptibility to ischemia and several seizure-inducing agents (Rudolphi et al. 1992, MacGregor and Stone 1994). This has been explained by their antagonism of adenosine A_1 receptors that indirectly potentiates NMDA-receptor-mediated Ca^{2+} entry to neurones (for review see Fredholm, 1997). However, although caffeine inhibits adenosine receptors in micromolar concentrations (Bruns et al. 1986), in our experiments systemic application of caffeine, resulting in 400 μM concentration in the brain, had no effect on NMDA-evoked $^{45}Ca^{2+}$ release. Moreover, Oyamada et al. (1998) recently demonstrated that 1-3 mM caffeine applied in the dialysis medium, significantly activates dopamine release in the rat striatum, and they related this effect to intracellular Ca^{2+} release, rather than to adenosine receptor antagonism.

The main result of this study, namely the lack of effect of systemically applied caffeine on the NMDA-induced $^{45}Ca^{2+}$ efflux in the rat DG, does not support the hypothesis that caffeine, administered systemically *in vivo* and present in brain in high micromolar concentrations, may potentiate CICR in the mammalian brain. Most probably higher, lethal doses of caffeine should be applied to obtain positive results.

The lack of effect of systemically applied caffeine on NMDA-evoked $^{45}Ca^{2+}$ release cannot be explained by possible simultaneous stimulation of RyR and inhibition of IP_3R. In theory, both these effects could cancel each other, resulting in net lack of changes in NMDA-evoked $^{45}Ca^{2+}$ release. However, DG granule neurones contain RyR, mostly RyR_2 isoform, whereas in the rat hippocampal formation IP_3R were found exclusively in the CA1 neurones (Sharp et al. 1993). Also, as we described previously, a pharmacological profile of the NMDA-evoked $^{45}Ca^{2+}$ release in DG strictly corresponds to CICR involving RyR channels (Łazarewicz et al. 1998).

In conclusion, in this *in vivo* study caffeine administered locally to the DG in a high, 50 mM concentration potentiated a spontaneous and NMDA-evoked $^{45}Ca^{2+}$ release in the rat DG. We attribute this effect to direct interference of caffeine with RyR, well known from *in vitro* studies. These positive results justify utilisation of microdialysis as a sensitive tool in studies on intracellular Ca^{2+} release in rat brain *in vivo*. Systemic application of caffeine in a pharmacologically active dose had no effect on NMDA-evoked $^{45}Ca^{2+}$ release in DG. Thus, our results confirm classical opinion that RyR in brain neurones are not susceptible to modulation by caffeine administered in pharmacologically relevant doses. Possibly, modulation of RyR by caffeine given in high micromolar concentrations may occur in ryanodine channels isolated and reconstituted in artificial phospholipid membranes (Sitsapesan and Williams 1990), or in non-mammalian RyR (Orkand and Thomas 1995), but not in mammalian central neurones.

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