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Somatostatin release by glutamate *in vivo* is primarily regulated by AMPA receptors

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1 We have used *in vivo* microdialysis in anaesthetized rats to investigate whether levels of striatal somatostatin (SRIF) can be increased in response to application of the ionotropic glutamate receptor agonists AMPA and NMDA.

2 Application of both AMPA and NMDA (10, 50, 100 and 500 μM) for 20 min periods produced concentration-dependent increases in the extracellular levels of SRIF. A 500 μM dose of each compound was shown to be the most potent concentration tested, increasing levels of SRIF by 32 fold (NMDA) and 35 fold (AMPA). At lower concentrations (10 μM) NMDA failed to evoke significant amounts of SRIF while AMPA increased levels of the peptide 2.3 fold.

3 Application of the respective receptor antagonists APV (NMDA receptor) and DNQX (AMPA receptor) abolished the abilities of the agonists to evoke release of SRIF. Interestingly DNQX abolished the ability of NMDA to evoke release of the peptide as well.

4 The ability of both AMPA and NMDA to evoke increases in the levels of extracellular SRIF further illustrates the reciprocal relationship that exists between SRIF and glutamate in the striatum which impacts particularly on dopaminergic functioning in this region.

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; APV, 2-amino-5-phosphopentanoic acid; DNQX, 6,7-dinitroquinoxaline-2,3-dione; EIA, enzyme immuno assay; NMDA, N-methyl-D-aspartate, SRIF, somatostatin

Introduction

Somatostatin (SRIF) is a cyclic tetradecapeptide first isolated from ovine hypothalami (Brazeau *et al*., 1973). Somatostatin and its receptors have a wide distribution in the brain (Schindler *et al*., 1966; 1997) the peptide has been shown to be stored in synaptic vesicles and released in a calcium-dependent manner (Epelbaum *et al*., 1977; Iversen *et al*., 1978). Five SRIF receptor genes have been sequenced and cloned and the complementary receptor proteins are termed sst_{1-5}, one of which, the sst_2 receptor, has a splice variant termed $sst_{2(b)}$ (Vanetti *et al*., 1992). The receptors are all composed of seven-transmembrane spanning units and are all coupled to G-proteins.

Within the striatum SRIF is found in a discrete population of medium-sized aspiny interneurones ($\sim$12–25 μm in diameter) which account for 1–2% of the total population of interneurones in this region (Kawaguchi *et al*., 1995). These SRIF containing interneurones also contain neuropeptide Y (Vincent & Johansson, 1983) and neuronal nitric oxide synthase (Kawaguchi *et al*., 1995), and receive direct glutamatergic cortical inputs (Vuillet *et al*., 1989). Whether these cells also contain γ-aminobutyric acid (GABA) is a matter of some debate because rats must be treated with colchicine before any detectable immunoreactivity for glutamic acid decarboxylase is seen (Vuillet *et al*., 1989; Kubota *et al*., 1993).

Application of SRIF to the striatum *in vivo* results in large increases in dopamine and GABA levels which are mediated *via* a glutamatergic mechanism (Hathway *et al*., 1998). Subsequent investigations have shown that these effects are mediated *via* the sst_2 receptor present on the terminals of cortico-striatal glutamatergic afferents (Hathway *et al*., 1999). From these results it would appear that there is a close relationship between SRIF and Glu and in the same way as SRIF releases Glu a reciprocal relationship may exist. Previously SRIF release has been observed *in vivo* in response to KC1 (Radke *et al*., 1993). The study described here investigated the ability of the ionotropic glutamate receptor agonists N-methyl-D-asparate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) to elicit increases in extracellular levels of SRIF in the rat striatum.

Methods

Adult male Wistar rats (250–300 g) were used in all experiments. They were anaesthetized with a 25% urethane solution and mounted in stereotaxic apparatus (Kopf Instruments, U.S.A.) *via* two ear bars and an incisor bar set at 7° above the interaural line. Incisions were made in the scalp and coordinates for microdialysis probe placement were calculated relative to bregma according to a stereotaxic atlas

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(Pellegrino *et al*., 1979). Microdialysis probes (Mettallhantering, Stockholm, Sweden) were placed in the medial portion of the right striatum at the coordinates 2 mm rostral to bregma, 2.5 mm lateral to the midline and 6 mm down from the surface of the brain. Body temperature was maintained using a homeothermic heated blanket (Harvard Apparatus, U.K.) at 37°C. All animal use procedures were in strict accordance with U.K. Home Office guidelines and specifically licensed under the Animals (Scientific Procedures) Act 1986.

Microdialysis sampling started 90 min after probe implantation. A Krebs–Ringer solution (pH 7.4) was made up with Milli-Q (Waters, Milford, MA, U.S.A.) deionized water (resistance 18.2 MΩ cm^{-1}) containing (mM): NaCl 138, $CaCl_2$ 1.5, $NaHCO_3$ 11, KCl 5, $MgCl_2$ 1, and NaH_2PO_4 1; 5 μM. This solution was passed through the probes at a rate of 2.0 μl min^{-1} using a syringe pump (CMA-10; CMA Microdialysis). Samples were collected from the outflow tube of the probe into 500 μl Eppendorf tubes containing 2 μl of 10% acetic acid at 20 min intervals using a fraction collector (CMA-142; CMA Microdialysis). A liquid switch (CMA-110; CMA Microdialysis) was placed between the syringe pump and the probe to switch between perfusion solutions without introducing air bubbles into the probe. The dead space between the switch and the end of the outflow tubing at the end of the probe was calculated to be 10.5 μl, and this was taken into account when switching between solutions. Collected samples (40 μl) were frozen (−20°C) immediately upon collection and subsequently used for enzyme immuno assay (EIA) analysis.

Ionotropic glutamate receptor agonists NMDA or AMPA were applied twice, for 20 min by retrodialysis, 60 and 120 min after the commencement of sampling. Four concentrations of each compound, 10, 50, 100 and 500 μM, were used, with the perfusing solution being returned to Krebs–Ringer following each administration. The ability of the effects of NMDA and AMPA to be antagonized was also investigated. The NMDA antagonist, 2-amino-5-phosphopentanoic acid (APV), and the AMPA/ kainate receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), were retrodialysed for 90 min before, and during, application of 100 μM NMDA and AMPA. To clarify that SRIF was released neuronally, tetrodotoxin (Sigma) was retrodialysed into the striatum for 60 min prior to a 100 μM AMPA challenge.

The enzyme immunoassay used for the measurement of endogenous somatostatin release was purchased from Peninsula Laboratories Inc. (Belmont, California, U.S.A.: part number EIAH-8001). A 10-point standard curve was prepared using known concentrations of somatostatin (0–10 ng ml^{-1}) and 40 μl of each concentration was added to each well. Following this, 40 μl of unknown sample plus 10 μl of assay buffer were pipetted to each well. Antisera (25 μl) was then added to each well followed by 25 μl of biotinylated peptide solution. The plate was gently agitated, sealed and left at room temperature for 2 h on a shaker table. At the end of this period the plate was washed thoroughly with buffer five times and 100 μl of a streptavidin–HRP/ buffer solution was added to each well. The plate was then resealed and incubated at room temperature for 60 min on a shaker table. The immunoplate was then washed five times in buffer and 100 μl of substrate solution (TMB and H_2O_2) was added to each well. The plate was then sealed and incubated on a shaker table for 30 min to allow the colour reaction to develop, which was stopped by the addition of 100 μl of 2N HCl. The bottom of the immunoplate was then cleaned with an ethanol soaked tissue and the plate read using a Microtiter Plate Reader at a wavelength of 450 nm. The specificity of the antibody used in the assay has been investigated by the manufacturers (Peninsula Laboratories Inc.).

In each experiment it was confirmed that the mean concentrations of SRIF measured in the three control samples taken before a drug challenge did not differ significantly from one another using a repeated-measures ANOVA. To assess the effects of drugs under the different conditions in each animal, the sample taken immediately before the drug administration was defined as 100%, and the change from this during the 20 min drug challenge was expressed as a percentage. In each case a repeated-measures ANOVA was carried out, and, where significant ($P<0.05$) *post hoc* comparisons made with Tukey's test.

At the end of each experiment rats were killed by cervical dislocation and their brains were rapidly dissected and sectioned to confirm accuracy of probe placements.

Results

Examination of the dissected brains revealed that in all animals the microdialysis probes were localized in the medial portion of the right striatum.

Administration of the glutamate receptor agonists, NMDA and AMPA, produced concentration-dependent increases in the levels of extracellular SRIF present in the striatum. The most potent concentration of each compound tested was the 500 μM dose. A 15 min challenge of NMDA (500 μM) potently stimulated release of SRIF increasing levels of the peptide 32.3 ± 8.6 fold ($P<0.01$) compared with baseline levels. A similar application of 500 μM AMPA increased levels of SRIF by 35.3 ± 1.9 fold ($P<0.01$) compared with baseline levels. A 15 min application of 100 μM NMDA significantly increased SRIF levels by 30.3 ± 13.6 fold ($P<0.01$), 50 μM NMDA significantly increased SRIF levels (10.6 ± 7 fold; $P<0.05$) while no effect on SRIF levels was observed when 10 μM NMDA was applied (0.7 ± 0.2 fold; Figure 1). Application of AMPA (100 μM) potently increased levels of SRIF by 14.2 ± 3.9 fold ($P<0.01$) with 50 μM AMPA increased SRIF levels 13.0 ± 9.3 fold ($P<0.05$) while 10 μM AMPA increased levels by 2.3 ± 0.5 fold ($P<0.05$; Figure 1). In all cases levels of SRIF had returned to basal levels in the sample immediately following application of the receptor agonists.

The NMDA receptor antagonist, APV, abolished NMDA-evoked SRIF release seen with the agonist (Figure 2). A 90 min treatment of APV (100 μM) decreased the effects of 100 μM NMDA (0.8 ± 0.09 fold compared with 30.3 ± 13.6 fold; $P<0.05$). A 90 min treatment of DNQX (100 μM) reduced evoked SRIF release to 1.5 ± 0.3 fold compared with 14.2 ± 3.9 fold ($P<0.01$) when 100 μM AMPA was applied. The effects of NMDA (100 μM) were also abolished by pre-treatment of the striatum with the AMPA/ kainate receptor antagonist DNQX (100 μM). Neither APV or DNQX had any effect on SRIF levels alone.

Application of tetrodotoxin (3 μM) abolished the ability of 100 μM AMPA to evoke increases in extracellular levels of SRIF (see Figure 2).

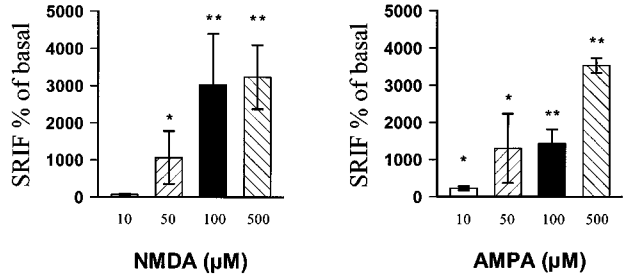

Figure 1 A figure illustrating the abilities of both NMDA (10, 50, 100 and 500 μM) and AMPA (10, 50, 100, 500 μM) to evoke increases in striatal SRIF levels. Basal levels of SRIF were 1.56 ± 0.40 ng ml^{-1} (combined means from all experiments). Values shown are the means of determinations from six animals $\pm$ s.e.mean $^*P<0.05$ $^{**}P<0.01$ indicate significant increase from baseline (100%) (Tukey's test). $F_{4,19} = 5.043$ (NMDA) $F_{4,19} = 25.229$ (AMPA).

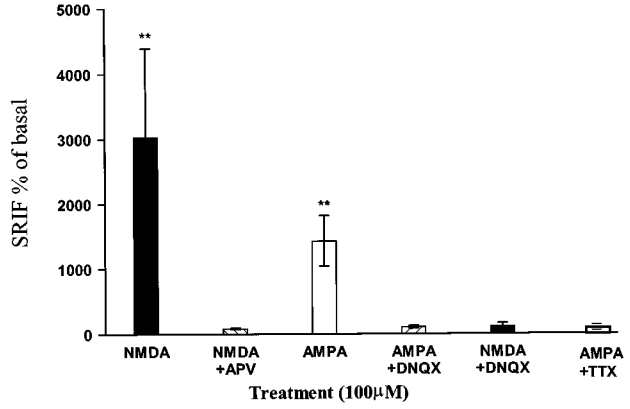

Figure 2 A figure illustrating the ability of APV (100 μM) and DNQX (100 μM) to abolish the effects of NMDA (100 μM) and AMPA (100 μM) in evoking SRIF release. Basal levels of SRIF were 1.80 ± 0.48 ng ml^{-1} (combined means from all experiments). TTX (3 μM) was utilized in this experiment. Bars indicate means $\pm$ s.e.mean with all values being determined from six animals. $^{**}P<0.01$ indicate significant increase from baseline (100%) (Tukey's test).

Discussion

The study described here demonstrates for the first time that somatostatin levels are increased *in vivo* in response to application of the ionotropic glutamate receptor agonists, AMPA and NMDA. Of the two compounds AMPA was shown to be the more potent at the lower concentrations tested while NMDA and AMPA were equipotent at the higher (500 μM) concentration tested. The effects of both of these compounds could be antagonized by the treatment of the tissue with the respective receptor antagonists, APV and DNQX. Interestingly the effects of NMDA could also be abolished by the presence of DNQX. Furthermore, application of tetrodotoxin (3 μM) abolished the ability of AMPA to evoke increases in SRIF levels illustrating that in these experiments SRIF was released neuronally presumably *via* vesicular exocytosis.

The degree of SRIF release was somewhat surprising, especially since physiologically equivalent levels of AMPA and NMDA were used. A study by Mathe *et al.* (1993) was unable to illustrate the release of the peptide to depolarizing challenges of KCl and veratridine, however Radke *et al.* (1993) were able to demonstrate this. The levels of release reported by Radke *et al.* (1993) were below those reported here, but the method of measurement of SRIF in these studies was different to that used in this study.

This study investigated the relationship between Glu and SRIF, which has been shown to be important in mediating the release of DA previously (Hathway *et al.*, 1998), by applying ionotropic Glu receptor agonists to the striatum. Application of AMPA 10 μM significantly increased levels of SRIF by $232 \pm 50\%$ ($P<0.05$) whereas a similar concentration of NMDA was ineffective. Chen *et al.* (1998) have shown that the subtype of ionotropic glutamate receptor expressed by the medium-sized aspiny interneurones in the striatum is mainly of the AMPA subtype. This probably explains the greater potency of AMPA over NMDA at this concentration in this study. However, at the other concentrations tested NMDA and AMPA were seen to be the equipotent. This ability of NMDA to evoke SRIF release may be due to release of Glu from the terminals of corticostriatal projection neurones which in turn acts to release SRIF *via* AMPA receptors. Indeed it has previously been shown by Kendrick *et al.* (1996) that treatment of the striatum with DNQX prevents the release of some classical neurotransmitters by NMDA suggesting that the actions of this agonist may not be entirely postsynaptic. It may be that effects previously attributed to NMDA are in fact due to NMDA evoked Glu which mediates its actions *via* AMPA/kainate receptors. It is therefore reasonable to expect the same relationship may exist with SRIF release e.g. application of NMDA leads to the release of SRIF *via* NMDA evoked Glu release. Indeed, in this study application of NMDA in the presence of the AMPA/kainate receptor antagonist DNQX failed to elicit a response. This phenomenon could be attributed to DNQX having an affinity for the glycine site of the NMDA receptor, however, the affinity of DNQX for this site is relatively low.

Both NMDA and AMPA have been shown previously to increase the release of SRIF from various tissues. Investigations of SRIF release have also been carried out in primary culture. Williams *et al.* (1991) investigated the effects of NMDA and kainate upon SRIF release from cultured medium-sized aspiny interneurones which, as already mentioned, contain SRIF together with other compounds. NMDA was able to elicit approximately a 400% increase in SRIF-like immunoreactivity (SRIF-LI), while kainate was slightly more potent, increasing levels by 500%. The effects of both NMDA and kainate were shown to be calcium-dependent. Also APV and MK-801 were able to prevent the NMDA-evoked increases but they could not prevent KCl induced increases in SRIF-LI. Fontana *et al.* (1996) investigated release of SRIF in cultured hippocampal neurones and found that both NMDA and AMPA could stimulate SRIF release in this preparation. The effects of both of these agonists could be blocked by MK-801 and DNQX, respectively, and were calcium-dependent. Only responses evoked by NMDA were sensitive to tetrodotoxin. However, the latter study did not find a significant difference in the abilities of NMDA and AMPA in eliciting SRIF release similar to the experiments described in this study. This group concluded that SRIF may be responsible for mediating some of the excitotoxic effects of Glu and Asp in the hippocampus and perhaps throughout the CNS.

The basal ganglia and inparticular the striatum, are known to be involved in movement disorders such as Parkinson's and Huntington's diseases. Here we have shown that SRIF release is under the control of glutamatergic activity. It may be that SRIF plays a role in the control of movement, future studies will investigate striatal deficits in sst_2 null mice.

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