British Journal of Pharmacology (1999) 127, 275–283
http://www.stockton-press.co.uk/bjp

Control of glutamate release by calcium channels and κ-opioid receptors in rodent and primate striatum

*,[1]M.P. Hill & [1]J.M. Brotchie

[1]Manchester Movement Disorders Laboratory, 1.124 Division of Neuroscience, School of Biological Sciences, Stopford Building, University of Manchester, Oxford Road, Manchester, M13 9PT

1 The modulation of depolarization (4-aminopyridine, 2 mM)-evoked endogenous glutamate release by κ-opioid receptor activation and blockade of voltage-dependent Ca^{2+}-channels has been investigated in synaptosomes prepared from rat and marmoset striatum.

2 4-Aminopyridine (4-AP)-stimulated, Ca^{2+}-dependent glutamate release was inhibited by enadoline, a selective κ-opioid receptor agonist, in a concentration-dependent and *nor*-binaltorphimine (*nor*-BNI, selective κ-opioid receptor antagonist)-sensitive manner in rat ($IC_{50} = 4.4 \pm 0.4$ μM) and marmoset ($IC_{50} = 2.9 \pm 0.7$ μM) striatal synaptosomes. However, in the marmoset, there was a significant (≈23%) *nor*-BNI-insensitive component.

3 In rat striatal synaptosomes, the Ca^{2+}-channel antagonists ω-agatoxin-IVA (P/Q-type blocker), ω-conotoxin-MVIIC (N/P/Q-type blocker) and ω-conotoxin-GVIA (N-type blocker) reduced 4-AP-stimulated, Ca^{2+}-dependent glutamate release in a concentration-dependent manner with IC_{50} values of 6.5 ± 0.9 nM, 75.5 ± 5.9 nM and 106.5 ± 8.7 nM, respectively. In marmoset striatal synaptosomes, 4-AP-stimulated, Ca^{2+}-dependent glutamate release was significantly inhibited by ω-agatoxin-IVA (30 nM, 57.6 ± 2.3%, inhibition), ω-conotoxin-MVIIC (300 nM, 57.8 ± 3.1%) and ω-conotoxin-GVIA (1 μM, 56.7 ± 2%).

4 Studies utilizing combinations of Ca^{2+}-channel antagonists suggests that in the rat striatum, two relatively distinct pools of glutamate, released by activation of either P or Q-type Ca^{2+}-channels, exist. In contrast, in the primate there is much overlap between the glutamate released by P and Q-type Ca^{2+}-channel activation.

5 Studies using combinations of enadoline and the Ca^{2+}-channel antagonists suggest that enadoline-induced inhibition of glutamate release occurs primarily *via* reduction of Ca^{2+}-influx through P-type Ca^{2+}-channels in the rat but *via* N-type Ca^{2+}-channels in the marmoset.

6 In conclusion, the results presented suggest that there are species differences in the control of glutamate release by κ-opioid receptors and Ca^{2+}-channels.

Keywords: Striatum; glutamate; κ-opioid; Ca^{2+}-channels; ω-agatoxin-IVA; ω-conotoxin-MVIIC; ω-conotoxin-GVIA

Abbreviations: ω-aga-IVA, ω-agatoxin-IVA; 4-AP, 4-aminopyridine; ω-con-GVIA, ω-conotoxin-GVIA; ω-con-MVIIC, ω-conotoxin-MVIIC; *nor*-BNI, *nor*-binaltorphimine; TTX, tetrodotoxin; VDCCs, voltage-dependent calcium channels

Introduction

Glutamatergic neurotransmission in the CNS is modulated by κ-opioid receptor activation. Thus, κ-opioid receptor activation inhibits glutamate release in the striatum (Hill & Brotchie, 1995), rat dorsal raphe nucleus (Pinnock, 1992a), rat locus coeruleus (Pinnock, 1992b), guinea-pig hippocampal mossy fibres (Gannon & Terrian, 1991; Weisskopf *et al*., 1993), rat cerebral cortex (Nicol *et al*., 1996) and rat substantia nigra pars reticulata (Maneuf *et al*., 1995). Furthermore, enadoline, a selective κ-opioid receptor agonist, has been shown to have anti-epileptic activity and neuroprotective properties similar to excitatory amino acid antagonists (Mackay *et al*., 1993; Kusumoto *et al*., 1992; Singh *et al*., 1990). A number of mechanisms have been suggested to modulate this inhibition. These include, inhibition (or reduced activation) of voltage-dependent Ca^{2+}-channels (VDCCs) (Cherubini & North, 1985; Gross & McDonald, 1987; Adamson *et al*., 1989; Xiang *et al*., 1990; Wiley *et al*., 1997), activation of a K^+ conductance (Shen & Crain, 1990; Grudt & Williams, 1993; Ma *et al*., 1995; Henry *et al*., 1995; Ikeda *et al*., 1995) and inhibition of Na^+-channels (Millan *et al*., 1995). The inhibition of Ca^{2+}-channels by κ-opioid-receptor activation is thought to be G-protein-mediated (Gross & Macdonald, 1987; Rhim & Miller, 1994; Wiley *et al*., 1997). In addition, a recent study demonstrated that κ-opioid receptor activation could modulate the activity of N-, P- and Q-type Ca^{2+}-channels (Wiley *et al*., 1997).

Several subtypes of VDCCs have been identified by pharmacological, electrophysiological and, more recently, molecular cloning techniques (Miller, 1992; Snutch & Reiner, 1992; Zhang *et al*., 1993). These can be divided into two main classes, high threshold of activation (N, P, Q and R-type) and low threshold of activation (T-type). Neurotransmitter release seems to be preferentially coupled to one or more of the high-threshold Ca^{2+}-currents (Leubke *et al*., 1993; Takahashi & Momiyama, 1993; Toth *et al*., 1993). These can be grouped into various classes according to their sensitivities to dihydropyridines (L-type) (Nowycky *et al*., 1985), ω-conotoxin GVIA (ω-con-GVIA; N-type) (Kasai *et al*., 1987) and ω-agatoxin IVA (ω-aga-IVA; P-type) (Mintz *et al*., 1992). Further subtypes of VDCCs have recently been pharmacologically identified in cerebellar granule neurons, including a channel sensitive to ω-conotoxin MVIIC (ω-con-MVIIC; Q-

*Author for correspondence; E-mail: m.hill@man.ac.uk

type) and a so called resistant (R-type) channel that is insensitive to all the Ca^{2+}-channel antagonists mentioned above (Ellinor *et al*., 1993; Wheeler *et al*., 1994). Studies in rat brain suggest that glutamate release is primarily dependent on Ca^{2+} influx through P-type channels (Mintz *et al*., 1992; Turner *et al*., 1992; Pocock & Nicholls, 1992; Burke *et al*., 1993; Kimura *et al*., 1995). Indeed, Kimura *et al*. (1995) demonstrated that the P-type Ca^{2+}-channel blocker ω-aga IVA almost totally blocked high potassium (K^+)-induced release of newly synthesized [^{3}H]-glutamate from rat striatal slices. However, in the rat, it has been reported that N- and Q-type Ca^{2+}-channels control glutamatergic neurotransmission at corticostriatal synapses (Lovinger *et al*., 1994). Furthermore, a recent study suggested that depolarization-induced glutamate release from rat striatal synaptosomes is controlled by Ca^{2+}-entry through N, P and Q-type Ca^{2+}-channels (Ambrosio *et al*., 1996). These studies suggest that at least three types of Ca^{2+}-channels can control presynaptic glutamate release in the striatum.

The aim of the present investigation was to determine the Ca^{2+}-channels involved in depolarization-induced glutamate release and in the modulation of glutamate release by κ-opioid receptor activation in rat and marmoset striatal synaptosomes.

Methods

Synaptosome preparation

Synaptosomes were prepared from the striata of male Sprague Dawley rats (280–320 g, Charles River) or the common marmoset (350–400 g, Manchester University closed colony) using a modification of the method of Gray & Whittaker (1962). In each experiment, rats were killed by cervical dislocation and whole striata from six rats dissected out on ice and used to prepare synaptosomes. Marmosets were killed by overdose of sodium pentobarbitone and the whole striatum from one marmoset dissected out on ice and used to prepare synaptosomes. The striata from six rats or one marmoset were then placed in 25 ml ice-cold 320 mM sucrose solution and homogenized using 12 strokes of a hand-held homogenizer. The homogenate was centrifuged at $1000 \times g_{av}$ for 15 min in a Beckman JA-20 rotor at 4°C. The supernatant was collected and centrifuged at $12{,}000 \times g_{av}$ for 25 min, using the same rotor. The resultant pellet was re-suspended in 7.5 ml ice-cold 320 mM sucrose and layered onto 15 ml of 800 mM sucrose and centrifuged at $12{,}000 \times g_{av}$ for 30 min. The final pellet was re-suspended in HEPES-buffered medium (HBM; containing (in mM) NaCl, 140, KCl, 5, HEPES, 20, $NaHCO_3$, 5, $MgCl_2$, 1, Na_2SO_4, 0.12, glucose, 10; pH 7.4 to a concentration of approximately 0.3 mg ml^{-1} protein and used in experiments.

Measurement of glutamate release

Glutamate release from striatal synaptosomes was assayed using the method of Nicholls *et al*. (1987). The method uses the fluorescent properties of NADPH formed by the metabolism of glutamate by glutamate dehydrogenase. An aliquot (≈0.3 mg protein) of resuspended synaptosomes was placed in a constantly-stirred thermostated (37°C) cuvette in a Perkin Elmer LS 50B spectrofluorimeter, and pre-incubated for 5 min with $NADP^+$ (1 mM), glutamate dehydrogenase (Sigma type IV, 50 U/cuvette) and $CaCl_2$ (1.3 mM). In experiments involving nominally Ca^{2+}-free conditions, $CaCl_2$ was replaced by EGTA (1.3 mM). Glutamate release was monitored continually by observing the formation of NADPH from $NADP^+$ using excitation and emission wavelengths of 340 nm and 460 nm respectively. The first 80 s of each experiment were used to assess baseline glutamate release. 4-Aminopyridine (4-AP, 2 mM) or vehicle was added at time t = 80 s and glutamate release monitored for a further 220 s. All drugs were added 5 min prior to the addition of 4-AP or vehicle. All data shown in graphs and tables are derived from the change from baseline glutamate release, which was calculated by integrating the fluorescence signal with respect to time, using a spreadsheet macro (Microsoft Excel).

In each experiment a concentration-fluorescence curve for glutamate (1–5 μM) was established and protein levels determined and these data were used to convert fluorescence data to concentration (nmol glutamate mg^{-1} protein). Ca^{2+}-independent release was subtracted from total release to give a measure of Ca^{2+}-dependent release in each experiment.

Protein determination

The protein determination assay was performed essentially as described by Bradford (1976) using Coomassie Brilliant Blue G-250 (Biorad). Coomassie Brilliant Blue G-250 reagent (2.5 ml) was added to 0.05 ml of the synaptosome preparation and mixed well. Absorption was measured at 590 nm, using a Perkin Elmer Lambda Bio spectrophotometer, between 2 and 60 min after mixing. Calibration was performed with bovine serum albumin at 10, 50, 100, 150 and 200 mg ml^{-1}.

Statistical analyses

Gaussian distributions of glutamate release were assumed throughout experiments. Differences between means compared by a one-way ANOVA followed by Tukey-Kramer multiple comparisons test, a one-way ANOVA followed by a Dunnett's test or an unpaired *t*-test, where appropriate. The level of significance for rejection of the null hypothesis was set at $P<0.05$. Concentration-response graphs were fitted using a non-linear curve-fitting routine ($Y = Min + (Max - Min)/(1 + 10^{((LogIC50-X)*HillSlope)})$ with weighting ($1/Y^2$), Graphpad Prism) and IC_{50} values interpolated from the curve.

Materials

ω-Aga-IVA, ω-con-MVIIC and ω-con-GVIA were obtained from Alomone Labs Ltd. (Israel). *nor*-Binaltorphimine, cyprodime and naltrindole were obtained from RBI. Enadoline was a generous gift from Parke-Davis Neuroscience Centre (Cambridge, U.K.). All other drugs were obtained from Sigma.

Results

General properties of 4-AP-stimulated glutamate release

Baseline glutamate release was 0.14 ± 0.03 and 0.16 ± 0.03 nmol mg^{-1} protein of which $29 \pm 2\%$ and $31 \pm 3\%$ was Ca^{2+}-dependent in rat and marmoset striatal synaptosomes, respectively. Incubation of synaptosomes with 4-aminopyridine (4-AP, 2 mM) in the presence of Ca^{2+} caused a significant increase in glutamate efflux above baseline release in rat and marmoset striatal synaptosomes. Experiments in nominally Ca^{2+}-free conditions (i.e. EGTA replaced $CaCl_2$ in the incubation medium) demonstrated that $89 \pm 5\%$ of 4-AP-stimulated glutamate release was Ca^{2+}-

dependent in the rat striatum ($P<0.001$ vs control, unpaired t-test). In marmoset striatum, $88\pm7\%$ of 4-AP-stimulated glutamate release was Ca^{2+}-dependent ($P<0.001$ vs control, unpaired t-test, Table 1). In subsequent experiments the Ca^{2+}-independent glutamate release was subtracted from total glutamate release. Pre-incubation of rat and marmoset striatal synaptosomes with TTX (1 μM; 5 min prior to 4-AP) confirmed, as in previous reports (Tibbs *et al*., 1989), that 4-AP-stimulated glutamate release is independent on Na^+-channel activation (Table 1).

Effect of enadoline on 4-aminopyridine-stimulated Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes

Enadoline inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release from rat and marmoset striatal synaptosomes in a concentration-dependent manner (Figure 1). The maximum inhibitions were 47.5 ± 3.1 and $69.3\pm2.1\%$, observed at 300 μM endaoline, and the IC_{50} values were $4.4+0.4$ and 2.9 ± 0.7 μM, for rat and marmoset, respectively. Hill slopes were not significantly different to unity. In the presence of the κ-opioid-receptor antagonist *nor*-BNI (5 μM), endoline (30 μM)-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent gluamate release was totally inhibited in rat and partially inhibited in marmoset striatal synaptosomes (Figure 2). However, naltrindole (20 μM) and cyprodime (10 μM), δ-opioid and μ-opioid receptor antagonists respectively, had no effect on enadoline-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat or marmoset striatal synaptosomes (Figure 2).

Effects of Ca^{2+}-channel antagonists on 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes

In rat striatal synaptosomes, ω-aga-IVA (0.1 – 300 nM), ω-con-MVIIC (1 – 3000 nM) and ω-con-GVIA (1 – 3000 nM) inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in a concentration-dependent manner. The IC_{50} values for ω-aga-IVA, ω-con-MVIIC and ω-con-GVIA were 6.5 ± 0.9, 75.5 ± 5.9 and 106.5 ± 8.7 nM, respectively (Figure 3a). Hill slopes were not significantly different to unity. Inhibition of L-type Ca^{2+}-channels with nifedipine (1 – 30 μM) had no significant effect on glutamate release from rat striatal synaptosomes. In marmoset striatal synaptosomes. ω-aga-IVA (30 nM), ω-con-GVIA (1 μM) and ω-con-MVIIC (300 nM) significantly inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release by 57.6 ± 2.3, 56.7 ± 2 and $57.8\pm3.1\%$, respectively (Figure 3b). Table 2 shows the effect of various combinations of ω-aga-IVA (30 nM), ω-con-GVIA (1 μM) and ω-con-MVIIC (300 nM) on 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes. In both rat and marmoset striatal synaptosomes, each

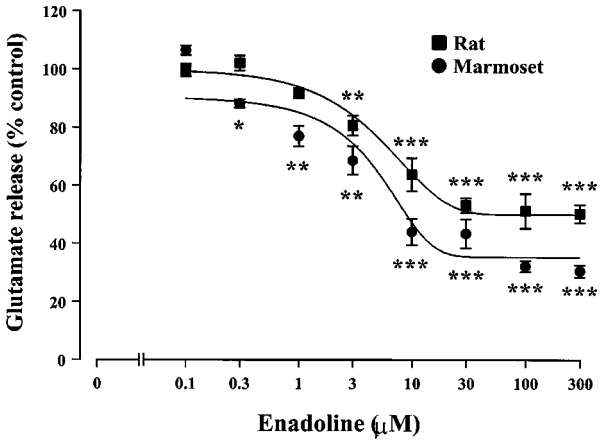

Figure 1 The κ-opioid receptor agonist, enadoline, inhibits 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes. To determine the effect of κ-opioid receptor activation on 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes, enadoline (0.1 – 300 μM) or vehicle was added to the synaptosome preparation 5 min prior to addition of 4-AP. The Ca^{2+}-dependent release of glutamate was then followed for a further 220 s. Vehicle effects have been subtracted from results. Values are expressed as mean ± s.e.mean; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $n=3-12$ experiments (one-way ANOVA, followed by Tukey-Kramer multiple comparisons test).

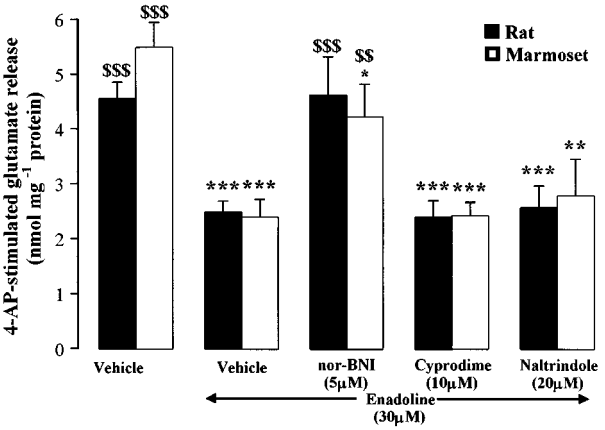

Figure 2 Enadoline-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes is mediated by κ-opioid receptor activation. To determine the receptor involved in enadoline-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes, nor-BNI (5 μM), cyprodime (10 μM), naltrindole (20 μM) or vehicle were added together with enadoline to the synaptosome preparation 5 min prior to addition of 4-AP. The Ca^{2+}-dependent release of glutamate was then followed for a further 220 s. Values are expressed as mean ± s.e.mean; $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared to vehicle, $\$\$P<0.01$, $\$\$\$P<0.001$ compared to enadoline + vehicle, $n=3-12$ experiments (one-way ANOVA, followed by Tukey-Kramer multiple comparisons test).

Table 1 4-AP-stimulated glutamate release is Ca^{2+}-dependent and requires Na^+-channel activation

Treatment	*Rat striatal synaptosomes glutamate release* (nmol mg^{-1} protein)	*Marmoset striatal synaptosomes glutamate release* (nmol mg^{-1} protein)
4-Aminopyridine (4-AP, 2 mM)	5.10 ± 0.3	6.24 ± 0.45
4-AP + EGTA (1.3 mM)	0.56 ± 0.1^a	0.75 ± 0.1^a
4-AP + tetrodotoxin (1 μM)	1.02 ± 0.2^a	1.34 ± 0.3^a

Values are expressed as the mean ± s.e.mean; $^aP<0.001$ compared to 4-AP-stimulated glutamate release $n=3-12$ experiments (unpaired t-test).

combination of antagonists significantly inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release. Furthermore, the level of inhibition was significantly greater than the individual antagonists when applied alone, except for the combination of ω-aga-IVA (30 nM) and ω-con-MVIIC (300 nM) in marmoset striatal synaptosomes.

Effects of enadoline on Ca^{2+}-channel antagonist-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes

The effects of combinations of enadoline and Ca^{2+}-channel antagonists on 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes are shown in Table 3. Co-incubation of enadoline (30 μM) and ω-aga-IVA (30 nM) significantly inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in both rat and marmoset striatal synaptosomes. However, in contrast to the marmoset, the level of inhibition was not significantly different to the effect of enadoline or ω-aga-IVA when applied alone in the rat. Co-incubation of enadoline (30 μM) and ω-con-GVIA (1 μM) significantly inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in both rat and marmoset striatal synaptosomes. However, in contrast to the rat, the level of inhibition was not significantly different compared to enadoline or ω-con-GVIA when applied alone in the marmoset striatum. Co-incubation of enadoline (30 μM) and ω-con-MVIIC (300 nM) significantly inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in both rat and marmoset striatal synaptosomes. The level of inhibition was significantly increased compared to enadoline or ω-con-MVIIC when applied alone in both rat and marmoset striatal synaptosomes.

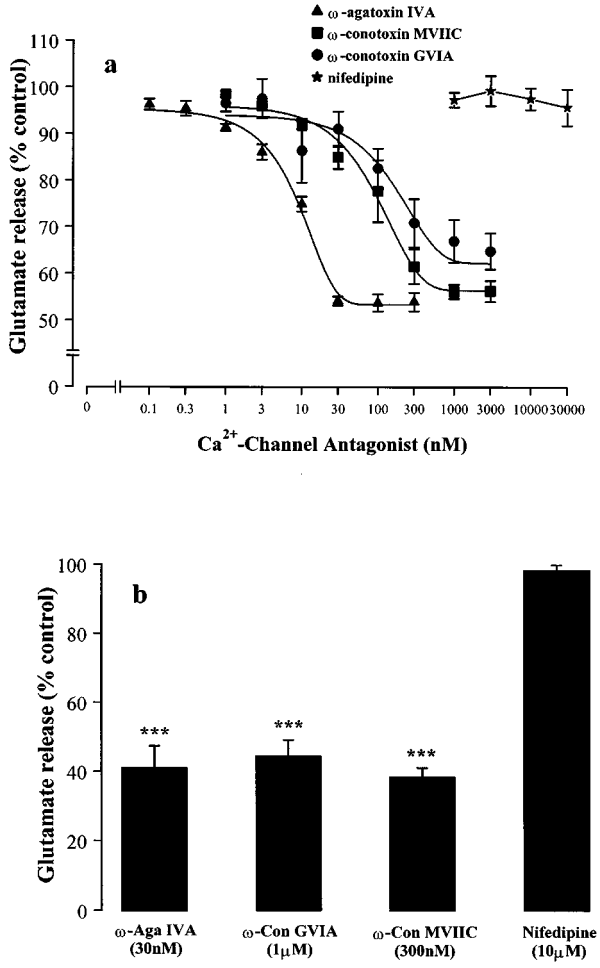

Figure 3 Ca^{2+}-channel antagonists inhibit 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes. To determine The effect of Ca^{2+}-channel antagonists on 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat (a) and (b) marmoset striatal synaptosomes, ω-aga-IVA (rat, 0.1–300 nM; marmoset, 30 nM), ω-con GVIA (rat, 1–3000 nM; marmoset 1 μM), ω-con-MVIIC (rat, 1–3000 nM; marmoset, 300 nM), nifedipine (rat, 1–30 μM; marmoset, 10 μM) or vehicle were added to the synaptosome preparation 5 min prior to addition of 4-AP. The Ca^{2+}-dependent release of glutamate was then followed for a further 220 s. Vehicle effects have been subtracted from results. Values are expressed as mean ± s.e.mean. (a) In rat striatal synaptosomes, each antagonist (apart from nifedipine) inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in a concentration-dependent manner (one-way ANOVA, followed by Tukey-Kramer multiple comparisons test; $n = 3-8$, significance levels have been omitted for clarity). (b) In marmoset striatal synaptosomes, each antagonist (apart from nifedipine) inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release, $^{***}P < 0.001$, compared to control, $n = 3-6$ experiments (one-way ANOVA, followed by Dunnett's test).

Discussion

General properties of 4-AP-stimulated glutamate release

Incubation of rat and marmoset striatal synaptosomes with 4-AP (2 mM) evoked a TTX and Ca^{2+}-dependent release of glutamate (Table 1). The TTX-sensitivity of 4-AP-stimulated glutamate release suggests a Na^+-channel-dependent release mechanism as has previously been reported (Tibbs *et al*., 1989). This mechanism is akin to that responsible for physiological neurotransmitter release. Previous studies have shown that millimolar concentrations of 4-AP can cause a large Ca^{2+}-independent release of neurotransmitters (Scheer & Lavoir, 1991; Carvalho *et al*., 1995). However, our data suggest that 2 mM 4-AP releases endogenous glutamate in a highly Ca^{2+}-dependent manner in both rat and marmoset striatal synaptosomes. The small amount of Ca^{2+}-independent glutamate release stimulated by 4-AP is likely to be a result of reversal of the glutamate transporter which normally carries glutamate and K^+ ions into the synaptosome in exchange for glutamate and Na^+ ions.

Modulation of 4-AP-stimulated, Ca^{2+}-dependent glutamate release by κ-opioid receptor activation

The present study confirms and extends our previous report (Hill & Brotchie, 1995), that enadoline inhibited 4-AP (2 mM)-stimulated Ca^{2+}-dependent glutamate release from rat and marmoset striatal synaptosomes in a concentration-dependent manner (Figure 1). Greater maximum inhibition was observed in synaptosomes prepared from primate (69.3%) than rat (47.5%) striatum. Gannon & Terrian (1991) demonstrated that U-50,488H, a selective κ-opioid receptor agonist inhibited 30 mM KCl stimulated Ca^{2+}-dependent glutamate release from guinea-pig hippocampal mossy fibre synaptosomes in a concentration-dependent manner (maximum of 77%). Similarly, the κ-opioid receptor agonist spiradoline was found to inhibit 46 mM KCl-stimulated Ca^{2+}-dependent glutamate release by 71% in rat cortical slices (Nicol *et al*., 1996). The differences in maximal inhibition by enadoline between species in the present study and between the effects of enadoline reported here in the rat and the effects of other κ-opioid receptors agonist in reducing glutamate release in previous studies may reflect a combination of species differences, different pools of glutamate being released by KCl compared

Table 2 The effect of combinations of ω-Aga-IVA ω-con-GVIA and ω-con-MVIIC on 4-AP-stimulated, Ca^{2+}-dependent glutamate release from rat and marmoset striatal synaptosomes

Toxins	*% Inhibition of 4-AP-stimulated glutamate release (rat)*	*Additive*	*% Inhibition of 4-AP-stimulated, glutamate release (marmoset)*	*Additive*
ω-Aga-IVA (30 nM)	45.9 ± 1.1^{a}	–	57.6 ± 2.3^{a}	–
ω-Con-GVIA (1 μM)	33.1 ± 5.4^{a}	–	56.7 ± 2.0^{a}	–
ω-Con-MVIIC (300 nM)	38.5 ± 8.7^{a}	–	57.8 ± 3.1^{a}	–
ω-Aga-IVA + ω-Con-GVIA	$78.8 \pm 7.2^{a,b,d}$	Totally	$88.2 \pm 7.1^{a,c,e}$	Partially
ω-Aga-IVA + ω-Con-MCIIC	$69.5 \pm 3.2^{a,b,h}$	Partially	51.7 ± 3.3^{a}	No
ω-Con-GVIA + ω-Con-MVIIC	$73.3 \pm 1.3^{a,d,g}$	Totally	$95.5 \pm 1.8^{a,d,f}$	Partially
ω-Aga-IVA + ω-Con-GVIA + ω-Con-MVIIC	$87.4 \pm 1.9^{a,b,d,f}$	Partially	$96.5 \pm 1.1^{a,b,d,f}$	Partially

Values are expressed as the mean ± s.e.mean; $^{a}P<0.001$ vs vehicle, $^{b}P<0.001$ compared to ω-aga-IVA alone, $^{c}P<0.01$ vs ω-aga-IVA alone, $^{d}P<0.001$ compared to ω-con-GVIA alone, $^{e}P<0.01$ vs ω-con-GVIA alone, $^{f}P<0.001$ vs ω-con-MVIIC alone, $^{g}P<0.01$ compared to ω-con-MVIIC alone, $^{h}P<0.05$ compared to ω-con-MVIIC alone; $n=3-9$ experiments (one-way ANOVA, followed by Tukey-Kramer multiple comparisons test).

Table 3 The effect of enadoline in combination with ω-aga-IVA, ω-con-GVIA and ω-con-MVIIC on 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat and marmoset striatal synaptosomes

Treatment	*% Inhibition of 4-AP-stimulated glutamate release (rat)*	*Additive*	*% Inhibition of 4-AP-stimulated, glutamate release (marmoset)*	*Additive*
Enadoline (30 μM)	46.8 ± 2.5^{a}	–	56.5 ± 5.0^{a}	–
ω-Aga-IVA (30 nM)	45.9 ± 4.6^{a}	–	57.6 ± 2.3^{a}	–
ω-Con-GVIA (1 μM)	33.1 ± 8.6^{a}	–	56.7 ± 2.0^{a}	–
ω-Con-MVIIC (300 nM)	38.5 ± 7.6^{a}	–	57.8 ± 3.1^{a}	–
ω-Aga-IVA + Enadoline	45.9 ± 2.7^{a}	No	$73.2 \pm 2.7^{a,b,d}$	Partially
ω-Con-GVIA + Enadoline	$60.4 \pm 3.3^{a,b,e}$	Partially	51.5 ± 1.7^{a}	No
ω-Con-MVIIC + Enadoline	$67.2 \pm 6.7^{a,b,f}$	Partially	$64.2 \pm 2.5^{a,c,g}$	Partially

Values are expressed as the mean ± s.e.mean; $^{a}P<0.001$ vs vehicle, $^{b}P<0.001$ vs enadoline alone, $^{c}P<0.01$ vs enadoline alone, $^{d}P<0.001$ vs ω-aga-IVA alone, $^{e}P<0.01$ vs ω-con-GVIA alone, $^{f}P<0.001$ vs ω-con-MVIIC alone; $^{g}P<0.05$ vs ω-con-MVIIC alone $n=4-12$ experiments (one-way ANOVA, followed by Tukey-Kramer multiple comparisons test).

to 4-AP, differences between brain regions and/or differences in tissue preparation (e.g. brain slices will contain postsynaptic elements and glia that could potentially modulate glutamate release). However, of interest is our observation that the enadoline (30 μM)-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat striatal synaptosomes was totally inhibited by the κ-opioid-receptor antagonist, *nor*-BNI, whereas it was only partially inhibited in the marmoset (Figure 2). The percentage of 4-AP-stimulated, Ca^{2+}-dependent glutamate release that is enadoline-sensitive and sensitive to nor-BNI is remarkably similar in both rat and marmoset. These data suggest that a portion of the enadoline-sensitive glutamate release in the marmoset striatum is not mediated by *nor*-BNI-sensitive κ-opioid receptors. A different subtype of κ-opioid receptor may be present in the marmoset striatum, which has a lower affinity for *nor*-BNI. The κ_2-opioid receptor has been shown to have a 100 fold lower affinity for nor-BNI, however, this receptor is also relatively insensitive to arylacetamide-like κ_1-opioid receptor ligands such as enadoline (Zukin *et al*., 1988; Clark *et al*., 1989). Unlike KCl-stimulated, Ca^{2+}-dependent glutamate release, 4-AP evokes release that is also dependent on Na^{+}-channel activation (Table 1). Direct inhibition of Na^{+}-channels by enadoline, as described by Millan *et al*. (1995) in the hippocampus, may play a role in enadoline-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in the marmoset. However, equivalent partial inhibition of Ca^{2+}-dependent glutamate release is seen whether release is stimulated by 4-AP or KCl (75%, data not shown), therefore, this mechanism is unlikely to account for the *nor*-BNI-insensitive portion of enadoline-sensitive glutamate release in the marmoset.

Ca^{2+}-channel subtypes mediating 4-AP-stimulated, Ca^{2+}-dependent glutamate release

The lack of effect on glutamate release of selective blockade of L-type Ca^{2+}-channels with nifedipine suggests that they are not involved in 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat or marmoset striatal synaptosomes (Figure 3a and b). This is in agreement with previous studies demonstrating that blockade of L-type channels has no effect on the release of various neurotransmitters (Pullar & Findlay, 1992; Mangano *et al*., 1991; Thomas *et al*., 1989). However, ω-con-GVIA (N-type blocker), ω-aga-IVA (P/Q-type blocker) and ω-con-MVIIC (N/P/Q-type blocker) inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in both rat and marmoset striatal synaptosomes. All the antagonists reduced glutamate release to a greater extent in the marmoset, than in the rat. However, as concentration-response curves were not performed in the marmoset, due to limitations of tissue availability, a full comparison of relative potency was not possible.

ω-Aga-IVA-sensitive glutamate release

Relatively selective blockers of P-type Ca^{2+}-channels have been identified (Mintz *et al*., 1992; Adams *et al*., 1993; Teramoto *et al*., 1993). One of these, ω-aga-IVA, is a peptide purified from the venom of the funnel web spider *Agelenopsis aperta* and has been reported to inhibit presynaptic glutamate release that is resistant to ω-con-GVIA (Turner *et al*., 1992). ω-Aga-IVA has been shown to block P-type channels with an estimated dissociation constant (K_D) of 0.7 nM and a forward

rate constant (k_{on}) of 2.4×10^5 M^{-1} s^{-1} (Mintz & Bean, 1993). Thus, at 30 nM ω-aga-IVA, 98% block of P-type channels could be expected in approximately 2 min. In addition, it has been demonstrated that concentrations of approximately 90 nM ω-aga-IVA are required to block Q-type current by 50% (Randall & Tsien, 1995) while concentrations up to 1 μM have no effect on N-type channels (Mintz *et al*., 1992). ω-Aga IVA inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat synaptosomes in a concentration-dependent manner with an IC_{50} of 6.5 ± 0.9 nM and maximum inhibition of $46.1 \pm 2\%$ (Figure 3a). In marmoset striatal synaptosomes, ω-aga IVA (30 nM) inhibited glutamate release by $57.6 \pm 2.3\%$ (Figure 3b). Therefore, these data strongly suggest that the release of glutamate is highly dependent on Ca^{2+}-entry through P-type channels. The IC_{50} obtained here is of the same order of magnitude, though slightly higher, than that reported for block of P-type current in cerebellar neurons (≈2 nM, Mintz *et al*., 1992; Randall & Tsien, 1995). However, there is a non-linear relationship between presynaptic Ca^{2+}-concentration and neurotranmitter release (Dodge & Rahamimoff, 1967; Augustine & Charlton, 1986). Therefore, the inhibition of glutamate release by ω-aga-IVA will not be directly proportional to the number of Ca^{2+}-channels blocked. Consequently, IC_{50} values for inhibition of glutamate release would be expected to be higher than those reported for direct inhibition of P-type Ca^{2+}-current. Indeed, it has been reported that ω-aga-IVA inhibits KCl-stimulated glutamate release from rat brain synaptosomes with an IC_{50} of 12.2 nM (Turner & Dunlap, 1995) and from rat striatal slices with an IC_{50} of 330 nM (Kimura *et al*., 1995). The maximum inhibition of glutamate release seen in the study of Kimura *et al*. (1995) was about 90%. This suggests that only P-type Ca^{2+}-channels are involved in KCl-evoked glutamate release in the striatum as blockade of N-type Ca^{2+}-channels had no effect. However, electrophysiological evidence suggests that neuronal transmission in a variety of synapses is controlled by more than one type of Ca^{2+}-channel (Takahashi & Momiyama, 1993). Furthermore, pharmacological studies have demonstrated that neurotransmission in a variety of synapses, including glutamatergic corticostriatal synapses, is controlled by more than one Ca^{2+}-channel (Turner *et al*., 1993; Graham & Burgoyne, 1995; Huston *et al*., 1995; Ambrosio *et al*., 1996; Harvey *et al*., 1996). Indeed, the IC_{50} value of 330 nM obtained by Kimura *et al*. (1995) may suggest a loss of selectivity of ω-aga-IVA for P-type channels and an action on Q-type channels (Mintz *et al*., 1992; Randall & Tsien, 1995). Another possible explanation for the high IC_{50} obtained by Kimura *et al*. (1995), as argued by Dunlap *et al*. (1994), is that the concentration of toxin at the synaptic site might be restricted by its ability to penetrate this region. This restricted entry to the synaptic site would not be a problem in a synaptosomal preparation, and therefore an apparent decreased potency might be expected in a slice preparation. Another possible reason for these differences in potency may be due to the different methods of stimulation of neurotransmitter release. Turner & Dunlap (1995) used a low (15 mM) concentration of KCl in superfused rat brain synaptosomes and Kimura *et al*. (1995) used a high (50 mM) concentration of KCl in rat striatal slices, in the present study, 4-AP (2 mM) was used to stimulate release from striatal synaptosomes. 4-AP stimulates neurotransmitter release by causing repetitive action potential firing in the synaptosomes (Tibbs *et al*., 1989) due to activation of Na^+-channels (Table 1), whereas high K^+ causes a stable depolarization. Luebke *et al*. (1993) recently described that the release of neurotransmitters from synaptosomes, induced by a brief stimulation of Na^+-channels with batrachotoxin, was less sensitive to blockade by ω-aga IVA, than that elicited by high K^+. Therefore, the role of P-type channel blockade in the study of Kimura *et al*. (1995) may be overestimated due to the use of high K^+ and the resultant non-physiological depolarization. Indeed, Kimura *et al*. (1995) demonstrated a significant effect of N-type channel blockade when the concentration of the K^+ pulse was reduced from 50 to 30 mM

ωCon-GVIA-sensitive glutamate release

The role of N-type Ca^{2+}-channels in 4-AP-stimulated, Ca^{2+}-dependent glutamate release was investigated with the selective irreversible N-type antagonist ω-con-GVIA (Kasai *et al*., 1987). ω-Con-GVIA is a peptide purified from the venom of the fish-hunting marine snail *Conus geographicus* (Olivera *et al*., 1994). In rat striatum, ω-con-GVIA significantly inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release with a maximum inhibition of $35.2 \pm 3.9\%$ (1 μM ω-con-GVIA) and an IC_{50} of 106.5 ± 8.7 nM (Figure 3a). This suggests that N-type calcium channels play a significant role in the control of glutamate release in the rat striatum, as ω-con-GVIA is selective for N-type channels at the concentrations used (Kasai *et al*., 1987). In marmoset striatal synaptosomes, ω-con-GVIA (1 μM) inhibited glutamate release by $56.7 \pm 2\%$ (Figure 3b), again suggesting a significant role for N-type Ca^{2+} channels. However, the magnitude of inhibition of glutamate release with ω-con-GVIA, in the rat and marmoset striatum, is higher than seen in some previous reports in mammalian brain (Kimura *et al*., 1995) and, as discussed earlier, may be due to the method of depolarization. Indeed, Luebke *et al*. (1993) demonstrated that the release of glutamate from hippocampal synaptosomes, induced by a brief stimulation of Na^+-channels, was more sensitive to blockade of ω-con GVIA, than that elicited by high KCl concentration. Furthermore, a previous report in which electrical depolarization of the corticostriatal pathway was employed showed ω-con-GVIA blocked about 50% of the activity at rat corticostriatal synapses (Lovinger *et al*., 1994), which more closely matches the results presented here.

ω-Con-MVIIC-sensitive glutamate release

ω-Con-MVIIC, a peptide from *Conus magnus* fish-hunting marine snail (Olivera *et al*., 1994) has been shown to block Ca^{2+}-channels termed Q-type channels. Q-type Ca^{2+}-channels are relatively resistant to ω-con-GVIA, ω-aga-IVA and dihydropyridine L-type blockers. However, it has been shown that ω-con-MVIIC binds to N-type channels with a 10–100 fold lower affinity than ω-con-GVIA and inhibits P-type channels at concentrations 100–1000 fold higher than ω-aga-IVA with slow on/off kinetics (Hillyard *et al*., 1992). Therefore, attributing an effect of ω-con-MVIIC to an action on Q-type Ca^{2+}-channels can be difficult. In the present study, ω-con-MVIIC inhibited 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat striatal synaptosomes in a concentration-dependent manner, with an IC_{50} of 75.5 ± 5.9 nM and maximum inhibition of $43.9 \pm 2.3\%$ (Figure 3a). This is in good agreement with the predicted IC_{50} for inhibition of Q-type Ca^{2+}-channels by ω-con-MVIIC (≈100 nM; Olivera *et al*., 1994). This suggests that ω-con-MVIIC-sensitive Ca^{2+}-channels are involved in the control of glutamate release in the rat striatum. In marmoset striatal synaptosomes, ω-con-MVIIC (300 nM) inhibited glutamate release by $57.8 \pm 3.1\%$ (Figure 3b), again supporting a major role for ω-con-MVIIC-sensitive Ca^{2+}-channels in the control of glutamate release in the marmoset striatum. A previous study demonstrated a

significant inhibition of Ca^{2+}-dependent glutamate release evoked by 30 mM KCl from rat brain synaptosomes of approximately 70% (Turner *et al*., 1995). This is higher than the inhibition reported here but may be due to the different area of brain being studied and the different method of depolarization. Another recent report demonstrated that ω-con-MVIIC blocked about 50% of excitatory synaptic transmission in rat neostriatal slices (Lovinger *et al*., 1994), which is similar to the results presented here.

Overlapping effects of Ca^{2+}-channel antagonists

From the data presented it can be seen that the sum totals of inhibitions by the Ca^{2+}-channel antagonists exceeds 100% in both rat and marmoset striatal synaptosomes. This suggests the possibility that different pools of glutamate may be released by Ca^{2+}-entry through many more than one class of Ca^{2+}-channel. To assess this idea, combinations of the Ca^{2+}-channel antagonists were used to assess any overlap in their effects on glutamate release.

The results suggest that, in the rat striatum, there is an overlap between ω-aga-IVA and ω-con-MVIIC-sensitive glutamate release as in combination there is not a complete additive effect (Table 2). This could mean that a Ca^{2+}-channel with sensitivity to both ω-aga-IVA and ω-con-MVIIC supports a proportion of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in the rat striatum. At the concentrations used ω-aga-IVA is thought to selectively inhibit P-type Ca^{2+}-channels (Mintz *et al*., 1992; Randall & Tsien, 1995) and ω-Con-MVIIC may inhibit N, P and Q-type Ca^{2+}-channels (Hillyard *et al*., 1992; Randall & Tsien, 1995). Therefore, the partial overlap is most likely a result of inhibition of P-type Ca^{2+}-channels by ω-con-MVIIC. Thus, separate pools of glutamate, released *via* Ca^{2+}-influx through P or Q-type Ca^{2+}-channels are likely to exist in the rat striatum. The totally additive effect of ω-con-GVIA and ω-con-MVIIC in the rat suggests that ω-con-MVIIC is not inhibiting N-type Ca^{2+}-channels at the concentrations used. An alternative explanation is that there is a pool of glutamate, the release of which is controlled by both P and Q-type Ca^{2+}-channels. This explanation implies co-localization of Ca^{2+}-channels on individual nerve terminals. Turner *et al*. (1993) also suggested that Ca^{2+}-channels controlling glutamate release co-exist on glutamatergic nerve terminals in the striatum. This arrangement of Ca^{2+}-channels may be beneficial for two reasons: (1) if one channel is not functioning the other channel may prevent complete block of neurotransmission (Turner *et al*., 1993) and (2) different Ca^{2+}-channels may be differentially modulated by endogenous substances (Mogul *et al*., 1993; Yawo & Chuhma, 1993; Vázquez & Sánchez-Prieto, 1997) allowing for a variety of mechanisms for presynaptic modulation of transmitter release. Indeed, Ambrosio *et al*. (1996) demonstrated that activation of adenosine A_1 receptors inhibits 4-AP-stimulated endogenous glutamate release in rat striatal synaptosomes by inhibiting Ca^{2+}-flux through P/Q-type Ca^{2+}-channels but not through N-type Ca^{2+}-channels. In contrast, our results demonstrate that activation of κ-opioid receptors inhibits 4-AP-stimulated endogenous glutamate release in rat striatal synaptosomes by inhibiting Ca^{2+}-flux through P, Q and N-type Ca^{2+}-channels. This suggests that *in vivo*, dynorphin, the endogenous ligand at κ-opioid receptors, may control a pool of glutamate that is insensitive to adenosine acting *via* A_1-receptors. The finding that the effects of ω-aga-IVA and ω-con-GVIA were completely additive suggests that two independent pools of glutamate exist, one released by Ca^{2+}-influx through P-type channels, the other by Ca^{2+}-influx through N-type channels. The data from combining all three toxins and lack of effect of nifedipine suggest that a Ca^{2+}-channel resistant to all the antagonists can support a fraction of 4-AP-stimulated, Ca^{2+}-dependent glutamate release in rat striatum. A Ca^{2+}-channel resistant to all antagonists has previously been described in rat cerebellar granule neurons and termed the R-type Ca^{2+}-channel (Ellinor *et al*., 1993; Wheeler *et al*., 1994). A recent study has also demonstrated that R-type Ca^{2+} currents can evoke transmitter release at a rat central synapse (Wu *et al*., 1998). However, until selective antagonists are available, the nature of the Ca^{2+}-channel that supports the toxin-resistant portion of glutamate release cannot be fully elucidated.

In the marmoset striatum, a similar situation was observed in that certain combinations of antagonists produced effects that were additive, while others were only partially additive with the effects of the individual toxins alone. However, the details of the overlap in the effects of toxins were subtly different. ω-Aga-IVA and ω-con-MVIIC inhibit the same portion of 4-AP-stimulated Ca^{2+}-dependent glutamate release, as the inhibition caused by the toxins in combination is no greater than that caused by either toxin alone (Table 2). This may suggest that Q-type Ca^{2+}-channels are not involved in supporting 4-AP-stimulated, Ca^{2+}-dependent glutamate release in the marmoset striatum, as ω-aga-IVA is thought to be selective for P-type Ca^{2+}-channels at concentrations used (Mintz *et al*., 1992; Randall & Tsien, 1995), or as discussed above there may be a pool of glutamate controlled by both P and Q-type Ca^{2+}-channels. Furthermore, both ω-aga-IVA and ω-con-MVIIC were only partially additive in combination with ω-con-GVIA, suggesting the existence of pools of glutamate controlled by both P/Q and N-type Ca^{2+}-channels. This contrasts with the situation described above in the rat, where separate P and N-type Ca^{2+}-channel-sensitive pools of glutamate are apparent. Thus, there may be different combinations of Ca^{2+}-channels co-localized on glutamatergic nerve terminals in the marmoset compared to the rat striatum. Alternatively, there may be a Ca^{2+}-channel subtype present in the marmoset striatum that is sensitive to all the toxin antagonists as has been described in chicken brain synaptosomes (Lundy *et al*., 1994). However, the possibility that the co-operation of two or more Ca^{2+}-channels is required for optimal glutamate release, with inhibition of either being sufficient to block glutamate release cannot be ruled out, and as described above, is attractive.

Interaction of κ-opioid receptors and Ca^{2+}-channels

In rat striatum, enadoline did not enhance ω-aga-IVA-induced inhibition of 4-AP-stimulated, Ca^{2+}-dependent glutamate release. These data suggest there is a predominant interaction between P-type Ca^{2+}-channels and κ-opioid receptors in the rat striatum. In contrast, enadoline further inhibited glutamate release in the presence of ω-con-GVIA and ω-con-MVIIC in a partially additive manner (Table 3). This suggests that enadoline inhibits glutamate release that is controlled by Ca^{2+}-entry through N-type and P/Q-type Ca^{2+}-channels. The partial additive effect suggests that enadoline inhibits a portion of glutamate release that is not sensitive to these toxins. Another conclusion must be that ω-con-GVIA and ω-con-MVIIC inhibit a fraction of glutamate release that is not sensitive to enadoline (Table 3). This suggests that there may be a heterogeneous distribution of κ-opioid receptors on glutamatergic nerve terminals in the rat striatum that are differentially controlled by different Ca^{2+}-channels. A possible explanation is that pathways arising from different cortical or

thalamic areas have κ-opioid receptors that are coupled to different combinations of Ca^{2+}-channels that mediate the inhibition of glutamate transmission. In marmoset striatum, the results suggest that a qualitatively different interaction occurs, with the predominant interaction of enadoline being with N-type Ca^{2+}-channels. However, enadoline is able to interact with glutamate release that is sensitive to ω-aga-IVA and ω-con-MVIIC, although both these toxins block a portion of glutamate release that is not sensitive to enadoline (Table 3).

Differences in affinity between rat and primate for the compounds used in this study might explain some of the data we have reported. However, no previous studies have reported such a difference. Understanding the mechanisms controlling glutamate release and the species differences will have important implications for modulating chemical signalling in the basal ganglia and in the development of new treatments for neurological disorders.

We are grateful to the Parkinson's Disease Society for funding the research and to the Parke-Davis Neuroscience Centre (Cambridge, U.K.) for the generous gift of enadoline.

References

ADAMS, M.E., MINTZ, I.M., REILY, M.D., THANABAL, V. & BEAN, B.P. (1993). Structure and properties of ω-aga-IVB, a new antagonist of P-type calcium channels. *Mol. Pharmac.,* **44,** 681–688.

ADAMSON, P., MANTZOURRIDID, T., XIANG, J.Z., HAJIMOHAM-MADREZA, I., BRAMMER, M.J. & CAMPBELL, I.C. (1989). α_2-Adrenergic, κ-opiate, and P_1-purinergic autoreceptors have mutually antagonist effects: a new regulatory mechanism? *J. Neurochem.,* **53,** 1077–1082.

AMBROSIO, A.F., MALVA, J.O., CARVALHO, A.P. & CARVALHO, C.M. (1996). Modulation of Ca^{2+} channels by activation of adenosine A_1 receptors in rat striatal glutamatergic nerve terminals. *Neurosci. Lett.,* **220,** 163–166.

AUGUSTINE, G.J. & CHARLTON, M.P. (1986). Calcium dependence of presynaptic calcium current and post-synaptic response at the squid giant synapse. *J. Physiol. (Lond.),* **381,** 619–640.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

BURKE, S.P., ADAMS, M.E. & TAYLOR, C.P. (1993). Inhibition of endogenous glutamate release from hippocampal tissue by Ca^{2+} channel toxins. *Eur. J. Pharmac.,* **238,** 383–386.

CARVALHO, C.M., FERREIRA, I.L., DUARTE, C.B., MALVA, J.O., TRETTER, L., ADAM-VIZI, V. & CARVALHO, A.P. (1995). Relation of $[Ca^{2+}]_i$ to dopamine release in striatal synaptosomes: role of Ca^{2+} channels. *Brain Res.,* **669,** 234–244.

CHERUBINI, E. & NORTH, R.A. (1985). μ and κ Opioids inhibit transmitter release by different mechanisms. *Proc. Natl. Acad. Sci. U.S.A.,* **82,** 1860–1863.

CLARK, J.A., LIU, L., PRICE, M., HERSH, B., EDELSON, M. & PASTERNAK, G.W. (1989). Kappa opiate receptor multiplicity: evidence for two U50,488-sensitive kappa 1 subtypes and a novel kappa 3 subtype. *J. Pharmacol. Exp. Ther.,* **251,** 461–468.

DODGE, Jr, F.A., RAHAMIMOFF, R. (1967). On the relationship between calcium concentration and the amplitude of the end-plate potential. *J. Physiol. (Lond.),* **189,** 90P–92P.

DUNLAP, K., LUEBKE, J.I. & TURNER, T.J. (1994). Identification of calcium channels that control neurosecretion [letter; comment] *Science,* **266,** 828–831.

ELLINOR, P.T., ZHANG, J-F., RANDALL, A.D., ZHOU, M., SCHWARZ, T.L., TSIEN, R.W. & HORNE, W.A. (1993). Functional expression of a rapidly inactivating neuronal calcium channel. *Nature,* **363,** 455–458.

GANNON, R.L. & TERRIAN, D.M. (1991). U-50,488H inhibits dynorphin and glutamate release from guinea pig hippocampal mossy fibre terminals. *Brain Res.,* **548,** 242–247.

GRAHAM, M.E. & BURGOYNE, R.D. (1995). Effects of calcium channel antagonists on calcium entry and glutamate release from cultured rat cerebellar granule cells. *J. Neurochem.,* **65,** 2517–2524.

GRAY, E.G. & WHITTAKER, V.P. (1962). The isolation of nerve endings from brain. An electron microscopic study of cell fragments divided by homogenisation and centrifugation. *J. Anat. (London),* **96,** 79–88.

GROSS, R.A. & MCDONALD, R.L. (1987). Dynorphin A selectively reduces a large transient (N-type) calcium current of mouse dorsal root ganglion neurons in cell culture. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 5469–5473.

GRUDT, T.J. & WILLIAMS, J.T. (1983). Kappa-opioid receptors also increase potassium conductance. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 11429–11432.

HARVEY, J., WEDLEY, S., FINDLAY, J.D., SIDELL, M.R. & PULLAR, I.A. (1996). omega-Agatoxin IVA identifies a single calcium channel subtype which contributes to the potassium-induced release of acetylcholine, 5-hydroxytryptamine, dopamine, gamma-aminobutyric acid and glutamate from rat brain slices. *Neuropharmacology,* **35,** 385–392.

HENRY, D.J., GRANDY. D.K., LESTER, H.A., DAVIDSON, N. & CHAVKIN, C. (1995). Kappa-opioid receptors couple to inwardly rectifying potassium channels when coexpressed by *Xenopus* oocytes. *Mol. Pharmacol.,* **47,** 551–557.

HILL, M.P. & BROTCHIE, J.B. (1995). Modulation of glutamate release by a κ-opioid receptor agonist in rodent and primate striatum. *Eur. J. Pharmacol.,* **281,** R1–R2.

HILLYARD, D.R., MONJE, V.D., MINTZ, I.M., BEAN, B.P., NADASDI, L., RAMACHANDRAN, J., MILJANICH, G., AZIMI-ZOONOOZ, A., MCINTOSH, J.M., CRUZ, L.J., IMPERIAL, J.S. & OLIVERA, B.M. (1992). A new *Conus* peptide ligand for mammalian presynaptic Ca^{2+} channels. *Neuron,* **9,** 69–77.

HUSTON, E., CULLEN, G.P., BURLEY, J.R. & DOLPHIN, A.C. (1995). The involvement of multiple calcium channel sub-types in glutamate release from cerebellar granule cells and its modulation by $GABA_B$ receptor activation. *Neuroscience,* **68,** 465–478.

IKEDA, K., KOBAYASHI, T., USUI, H. & KUMANISHI, T. (1995). Functional couplings of the delta- and the kappa-opioid receptors with the G-protein-activated K^+ channel. *Biochem. Biophys. Comm.,* **208,** 302–308.

KASAI, H., AOSAKI, T. & FUKUDA, J. (1987). Presynaptic Ca^{2+} antagonist ω-conotoxin irreversibly blocks N-type Ca^{2+} channels in chick sensory neurons. *Neurosci. Res.,* **4,** 228–235.

KIMURA, M., YAMANISHI, Y., HANADA, T., KAGAYA, T., KUWADA, M., WATANABE, T., KATAYAMA, K. & NISHIZAWA, Y. (1995). Involvement of P-type calcium channels in high potassium-elicited release of neurotransmitters from rat brain slices. *Neuroscience,* **66,** 609–615.

KUSUMOTO. K., MACKAY, K.B. & MCCULLOCH, J. (1992). The effect of the kappa-opioid receptor against CI-977 in a rat model of focal cerebral ischaemia. *Brain Res.,* **576,** 147–151.

LOVINGER, D.M., MERRITT, A. & REYES, D. (1994). Involvement of N- and non-N-type calcium channels in synaptic transmission at corticostriatal synapses. *Neuroscience,* **62,** 31–40.

LUEBKE, J.I., DUNLAP, K. & TURNER, T.J. (1993). Multiple calcium channel types control glutamatergic synaptic transmission in the hippocampus. *Neuron,* **11,** 895–902.

LUNDY, P.M., HAMILTON, M.G. & FREW, R. (1994). Pharmacological identification of a novel Ca^{2+} channel in chicken brain synaptosomes. *Brain Res.,* **18,** 204–210.

MA, G.H., MILLER, R.J., KUZNETSOV, A. & PHILIPSON, L.H. (1995). Kappa-opioid receptor activates an inwardly rectifying K^+ channel by a G protein-linked mechanism: coexpression in *Xenopus* oocytes. *Mol. Pharmacol.,* **47,** 1035–1040.

MACKAY, K.B., KUSUMOTO, K., GRAHAM, D.I. & MCCULLOCH, J. (1993). Focal cerebral ischemia in the cat: pretreatment with a kappa-1 opioid receptor agonist, CI-977. *Brain Res.,* **618,** 213–219.

MANEUF, Y.P., MITCHELL, I.J., CROSSMAN, A.R., WOODRUFF, G.N. & BROTCHIE, J.M. (1995). Functional implications of kappa opioid receptor-mediated modulation of glutamate transmission in the output regions of the basal ganglia in rodent and primate models of Parkinson's disease. *Brain. Res.,* **683,** 102–108.

MANGANO, T.J., PATEL, J., SALAMA, A. & KEITH, R.A. (1991). Inhibition of K^+-evoked D-[^{3}H]Aspartate release and neuronal calcium influx by verapamil, diltiazem and dextrometorphan: evidence for non-L/non-N voltage-sensitive calcium channels. *Eur. J. Pharmacol.,* **192,** 9–17.

MILLAN, M.H., CHAPMAN, A.G. & MELDRUM, B.S. (1995). Dual inhibitory action of enadoline (CI977) on release of amino acids in the rat hippocampus. *Eur. J. Pharmacol.* **279,** 75–81.

MILLER, R.J. (1992). Voltage-sensitive Ca^{2+} channels. *J. Biol. Chem.,* **267,** 1403–1406.

MINTZ, I.M. & BEAN, B.P. (1993). Block of calcium channels in rat neurons by synthetic omega-aga-IVA. *Neuropharmacology,* **32,** 1161–1169.

MINTZ, I.M., VENEMA, V.J., SWIDEREK, K., LEE, T., BEAN, B.P. & ADAMS, M.E. (1992). P-type calcium channels blocked by the spider toxin ω-Aga-IVA. *Nature,* **355,** 827–829.

MOGUL, D.J., ADAMS, M.E. & FOX, A.P. (1993). Differential activation of adenosine receptors decreases N-type but potentials P-type Ca^{2+} current in hippocampal CA3 neurons. *Neuron,* **10,** 327–334.

NICHOLLS, D.G., SIHRA, T.S. & SANCHEZ-PRIETO, J. (1987). Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. *J. Neurochem.,* **49,** 50–57.

NICOL, B., ROWBOTHAM, D.J. & LAMBERT, D.G. (1996). Mu- and kappa-opioid inhibit K+ evoked glutamate release from rat cerebrocortical slices. *Neurosci. Lett.,* **218,** 79–82.

NOWYCKY, M.C., FOX, A.P. & TSIEN, R.W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature,* **316,** 440–443.

OLIVERA, B.M., MILJANICH, G.P., RAMACHANDRAN, J. & ADAMS, M.E. (1994). Calcium channel diversity and neurotransmitter release: the ω-conotoxins and ω-agatoxins. *Ann. Rev. Biochem.,* **63,** 823–867.

PINNOCK, R.D. (1992a). A highly selective kappa-opioid receptor agonist, CI-977, reduces excitatory synaptic potentials in the rat locus coeruleus *in vitro*. *Neuroscience,* **47,** 87–94.

PINNOCK, R.D. (1992b). Activation of kappa-opioid receptors depresses electrically evoked excitatory postsynaptic potentials on 5-HT-sensitive neurones in the rat dorsal raphe nucleus *in vitro*. *Brain Res.,* **583,** 237–246.

POCOCK, J.M. & NICHOLLS, D.G. (1992). A toxin (Aga-GI) from the venom of the spider *Agelenopsis aperta* inhibits the mammalian presynaptic Ca^{2+} channel coupled to glutamate exocytosis. *Eur. J. Pharmacol.,* **226,** 343–350.

PULLAR, I.A. & FINDLAY, J.D. (1992). Effect of voltage-sensitive calcium channel antagonists on the release of 5-hydroxytryptamine from rat hippocampus *in vivo*. *J. Neurochem.,* **59,** 553–559.

RANDALL, A. & TSIEN, R.W. (1995). Pharmacological dissection of multiple types of Ca2+ channel currents in rat cerebellar granule neurons. *J. Neurosci.* **4,** 2995–3012.

RHIM, H. & MILLER, R.J. (1994). Opioid receptors modulate diverse types of calcium channels in the nucleus tractus solitarius of the rat. *J. Neurosci.,* **14,** 7608–7615.

SCHEER, H.W. & LAVOIE, P.A. (1991). Mechanism of aminopyridine-induced release of [^{3}H]dopamine from rat brain synaptosomes. *Gen. Pharmacol.,* **22,** 169–172.

SHEN, K.F. & CRAIN, S.M. (1990). Dynorphin prolongs the action of potential of mouse sensory ganglion neurons by decreasing a potassium conductance whereas another specific kappa opioid does so by increasing a calcium conductance. *Neuropharmacology,* **29,** 343–349.

SINGH, L., VASS, C.A., HUNTER, J.C., WOODRUFF, G.N. & HUGHES, J. (1990). The anticonvulsant action of CI-977, a selective kappa-opioid receptor agonist: a possible involvement of the glycine/NMDA receptor complex. *Eur. J. Pharmacol.,* **191,** 477–480.

SNUTCH, T.P. & REINER, P.B. (1992). Ca^{2+} channels: diversity of form and function. *Curr. Opin. Neurobiol.,* **2,** 247–253.

TAKAHASHI, T. & MOMIYAMA, A. (1993). Different types of calcium channels mediate central synaptic transmission. *Nature,* **366,** 156–158.

TERAMOTO, T., KUWADA, M., NIIDOME, T., SAWADA, K., NISHIZAWA, Y. & KATAYAMA, K. (1993). A novel peptide from funnel web spider venom, ω-Aga-TK, selectively blocks P-type calcium channels. *Biochem. Biophys. Comm.,* **196,** 134–140.

THOMAS, J., FEUERSTEIN, T.J., DOOLEY, D.J. & SEEGER, W. (1989). Inhibition of norepinephrine and acetylcholine release from human neocortex by ω-conotoxin GVIA. *J. Pharmac. Exp. Ther.,* **252,** 778–785.

TIBBS, G.R., BARRIE, A.P., VAN MIEGHEM, F.J.E., MCMAHON, H.T. & NICHOLLS, D.G. (1989). Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: Effects on cytosolic free Ca^{2+} and glutamate release. *J. Neurochem.,* **53,** 1693–1699.

TOTH, P.T., BINDOKAS, V.P., BLEAKMAN, D., COLMERS, W.F. & MILLER, R.J. (1993). Mechanisms of pre-synaptic inhibition by neuropeptide Y at sympathetic nerve terminals. *Nature,* **364,** 635–641.

TURNER, T.J., ADAMS, M.E. & DUNLAP, K. (1992). Ca^{2+} channels coupled to glutamate release identified by ω-Aga IVA. *Science,* **258,** 310–316.

TURNER, T.J., ADAMS, M.E. & DUNLAP, K. (1993). Multiple Ca^{2+} channels types coexist to regulate synaptosomal neurotransmitter release. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 9518–9522.

TURNER, T.J. & DUNLAP, K. (1995). Pharmacological characterization of presynaptic calcium channels using subsecond biochemical measurements of synaptosomal neurosecretion. *Neuropharmacol.,* **34,** 1469–1478.

TURNER, T.J., LAMPE, R.A. & DUNLAP, K. (1995). Characterization of presynaptic calcium channels with omega-conotoxin MVIIC and omega-grammotoxin SIA: role for a resistant calcium channel type in neurosecretion. *Mol. Pharmacol.,* **47,** 348–353.

VÁZQUEZ, E & SÁNCHEZ-PRIETO, J. (1997). Presynaptic modulation of glutamate release targets different calcium channels in rat cerebrocortical nerve terminals. *Eur. J. Neurosci.,* **10,** 2009–2018.

WEISSKOPF, M.G., ZALUTSKY, R.A. & NICOLL, R.A. (1993). The opioid peptide dynorphin mediates heterosynaptic depression of hippocampal mossy fibre synapses and modulates long-term potentiation. *Nature,* **362,** 423–427.

WHEELER, D.B., RANDALL, A.D. & TSIEN, R.W. (1994). Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science,* **264,** 107–111.

WILEY, J.W., MOISES, H.C., GROSS, R.A. & MACDONALD, R.L. (1997). Dynorphin A-mediated reduction in multiple calcium currents involves a G(o) alpha-subtype G protein in rat primary afferent neurons. *J. Neurophysiol.,* **77,** 1338–1348.

WU, L.G., BORST, J.G. & SAKMANN, B. (1998). R-type Ca^{2+} currents can evoke transmitter release at a rat central synapse. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 4720–4725.

XIANG, J.Z., ADAMSON, P., BRAMMER, M.J. & CAMPBELL, I.C. (1990). The κ-opiate agonist U50488H decreases the entry of ^{45}Ca into rat cortical synaptosomes by inhibiting N- but not L-Type calcium channels. *Neuropharmacol.,* **29,** 439–444.

YAWO, H. & CHUHMA, N. (1993). Preferential inhibition of ω-conotoxin-sensitive presynaptic Ca^{2+} channels by adenosine autoreceptors. *Nature,* **365,** 256–258.

ZHANG, J.-F., RANDALL, A.D., ELLINOR, P.T., HORNE, W.A., SATHER, W.A., TANABE, T., SCHWARTZ, T.L. & TSIEN, R.W. (1993). Distinctive pharmacology and kinetics of cloned neuronal Ca^{2+} channels and their possible counterparts in mammalian CNS neurons. *Neuropharmacology,* **32,** 1075–1088.

ZUKIN, R.S., EGHBALI, M., OLIVE, D., UNTERWALD, E.M. & TEMPEL, A. (1988). Characterization and visualization of rat and guinea pig brain kappa opioid receptors: evidence for kappa 1 and kappa 2 opioid receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **85,** 4061–4065.

(Received December 5, 1998
Revised February 3, 1999
Accepted February 10, 1999)