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Effects of Ca^{2+} channel antagonists on striatal dopamine and DOPA release, studied by *in vivo* microdialysis

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1 To elucidate the mechanisms regulating the release of striatal dopamine and its precursor, 3,4-dihydroxyphenylalanine (DOPA), we determined the effects of various Ca^{2+} channel antagonists, an N-type Ca^{2+} channel antagonist, ω-conotoxin GVIA, a P-type Ca^{2+} channel antagonist, ω-agatoxin IVA, and a Q-type Ca^{2+} channel antagonist, ω-conotoxin MVIIC, on the basal and Ca^{2+}- and K^+-evoked release of striatal dopamine and DOPA, by use of *in vivo* microdialysis.

2 ω-Conotoxin GVIA strongly inhibited striatal basal dopamine release (IC_{50} = 0.48 nM), whereas this toxin only weakly modulated basal striatal DOPA release (IC_{50} = 9.55 nM). Neither ω-agatoxin IVA nor ω-conotoxin MVIIC affected the basal striatal release of dopamine and DOPA.

3 ω-Conotoxin GVIA strongly inhibited Ca^{2+}-evoked striatal dopamine release (IC_{50} = 0.40 nM), whereas Ca^{2+}-evoked striatal DOPA release only was weakly modulated (IC_{50} = 10.51 nM). Neither ω-agatoxin IVA nor ω-conotoxin MVIIC affected the Ca^{2+}-evoked release of striatal dopamine and DOPA.

4 Both ω-agatoxin IVA and ω-conotoxin MVIIC inhibited the K^+-evoked release of striatal dopamine (IC_{50} of ω-agatoxin IVA = 2.65 nM; IC_{50} of ω-conotoxin MVIIC = 12.54 nM) and DOPA (IC_{50} of ω-agatoxin IVA = 0.15 nM; IC_{50} of ω-conotoxin MVIIC = 3.05 nM), whereas ω-conotoxin GVIA had no effect on the K^+-evoked release of striatal dopamine and DOPA.

5 An increase in the extracellular Ca^{2+} and K^+ concentrations (Ca^{2+}- and K^+-evoked stimulation) did not affect tyrosine hydroxylase activity *in vivo*.

6 These findings suggest that striatal DOPA release is neurotransmitter-like and that, unlike the mechanisms of striatal dopaminergic transmission, this striatal DOPA transmission is at least partly regulated by voltage-sensitive Ca^{2+} channels.

Keywords: ω-Agatoxin IVA; ω-conotoxin GVIA; ω-conotoxin MVIIC; 3,4-dihydroxyphenylalanine; dopamine; striatum

Introduction

Whether or not L-3,4-dihydroxyphenylalanine (L-DOPA), which is a metabolic precursor of dopamine, is a neurotransmitter has been the subject of considerable scientific discussion (Misu & Coshima, 1993; Yue *et al*., 1993; Misu *et al*., 1995a) since L-DOPA administration has been shown to produce both excitatory (Misu & Goshima, 1993; Misu *et al*., 1995a) and toxic side effects (Misu & Goshima, 1993; Misu *et al*., 1995a,b; Basma *et al*., 1995), despite its clinical efficacy against Parkinson's disease and neuroleptic malignant syndrome (Otani *et al*., 1991a,b). If the mechanisms affecting the transmission of DOPA were clarified, then the pharmacological effects of DOPA in the central nervous system would be understood.

Synaptic neurotransmission is mediated by the entry of Ca^{2+} into presynaptic nerve terminals through voltage-sensitive, ion-specific channels (Smith & Augustine, 1988; Mulkey & Zucker, 1991; Okada *et al*., 1992; 1996a), of which different types have been shown to exist (Turner *et al*., 1993; Zhang *et al*., 1993). Electrophysiological experiments have demonstrated the existence of at least seven subtypes of voltage-sensitive Ca^{2+} channels (L-, N-, O-, P-, Q-, R- and T-type Ca^{2+} channels; Olivera *et al*., 1994; Randall & Tsien, 1995). Striatal dopamine transmission is regulated by the N-type Ca^{2+} channel (Zhang *et al*., 1995; Harvey *et al*., 1996), which is blocked by ω-conotoxin GVIA (Williams *et al*., 1992), but not by L- (Westerink *et al*., 1988; Pani *et al*., 1990) or T-type (Kato *et al*., 1992) Ca^{2+} channels. Recently, it has been suggested that the P-type Ca^{2+} channel (Takahashi & Momiyama, 1993; Kimura *et al*., 1995), which is blocked by ω-agatoxin IVA (Randall & Tsien, 1995), and the Q-type Ca^{2+} channel (Wheeler *et al*., 1994), which is blocked by ω-conotoxin MVIIC (Randall & Tsien, 1995), modulate synaptic transmission, although the functions of these subtypes have not yet been clarified *in vivo*. The actions of R- and O-type Ca^{2+} channels on central synaptic transmissions is still unclear, because selective inhibitors of these channels have not yet been discovered (Olivera *et al*., 1994; Randall & Tsien, 1995).

It is well known that dopamine is one of the most important classical neurotransmitters, since it has been confirmed that dopamine has typical neurotransmission mechanisms: tetrodotoxin (TTX) sensitivity, Ca^{2+}-dependence and K^+-induced release (Westerink *et al*., 1988; 1989). Misu and colleagues have demonstrated the neurotransmitter-like release of striatal DOPA (Misu & Goshima, 1993; Misu *et al*., 1995a,b). Nevertheless, it is unclear which subtypes of voltage-sensitive Ca^{2+} channels modulate the Ca^{2+}- and K^+-evoked release of DOPA and dopamine. Therefore, to investigate which voltage-sensitive Ca^{2+} channels are involved in basal, and Ca^{2+}- and K^+-evoked release of striatal dopamine and DOPA, ω-conotoxin GVIA, ω-conotoxin MVIIC and ω-agatoxin IVA were used for *in vivo* microdialysis.

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Methods

Male Wistar rats (Clea, Japan), weighing 250–300 g ($n=156$), were housed under conditions of constant temperature ($25\pm2°C$) with a 12 h light-dark cycle (Okada *et al*., 1997d). The experimental protocols used in this study were approved by the ethical committee of Hirosaki University.

Microdialysis system preparation

Each rat was anaesthetized with diethylether and placed in a stereotaxic frame. A concentric I-type dialysis probe (0.22 mm diameter; 3 mm exposed membrane; Eicom Co., Japan) was implanted into the striatum (A = 0.2 mm, L = 3.4 mm, V = −3.0 mm relative to the bregma) and the perfusion experiments were commenced 24–36 h after the rats had recovered from anaesthesia. The perfusion rate was always 1 μl min^{-1}, with modified Ringer solution composed of (in mM) Na^+ 145, K^+ 2.7, Ca^{2+} 1.2, Mg^{2+} 1.0, Cl^- 154.4, ascorbate 0.2 and buffered with 2 mM phosphate buffer and 1.1 mM Tris buffer to adjust the pH to 7.40 (Okada *et al*., 1997a,b,c). To study Ca^{2+}-independent release we used Ca^{2+}-free modified Ringer solution, in which the Ca^{2+} and the appropriate amount of Na^+ were replaced by 40 mM Mg^{2+} (Westerink *et al*., 1988; Okada *et al*., 1996a). To study the effects of an increase in the extracellular Ca^{2+} or K^+ level (Ca^{2+}- or K^+-evoked stimulation) on striatal dopamine and DOPA release, modified Ringer solution containing 3.4 mM Ca^{2+} or 50 mM K^+ was infused for 20 min. The ionic composition was modified and isotonicity was maintained by an equimolar decrease of Na^+ (Okada *et al*., 1996a). To study the effects of an increase in the extracellular level of Ca^{2+} or K^+ on tyrosine hydroxylase activity *in vivo*, striata were perfused with modified Ringer solution containing either 1 or 10 μM NSD1015 (m-hydroxybenzylhydrazine), which is a central aromatic amino acid decarboxylase inhibitor (Westerink *et al*., 1990; Okada *et al*., 1995; 1997b). Dialysate was injected automatically every 20 min into a high-performance liquid chromatograph equipped with an electrochemical detector (h.p.l.c.-e.d.) system by an autoinjector (Okada *et al*., 1992; 1996a; 1997c,d).

H.p.l.c.-e.d. system conditions for measuring extracellular levels of dopamine and DOPA

The h.p.l.c. system used for determination of the extracellular dopamine and DOPA levels was equipped with an e.d. (ECD-100, Eicom Co., Japan) and a graphic carbon electrode set at +600 mV (versus an Ag/AgCl reference electrode). The analytical column (Prospher RP-18: 70 mm × 4 mm internal diameter, particle size 5 μm) was purchased from Kanto Kagaku Co. (Japan). The mobile phase was 0.2 M citrate/0.02 M sodium acetate buffer, containing 2% (v/v) methanol, 150 mg l^{-1} octansulphonic sodium and 0.1 mM ethylenediaminetretraacetic acid-2Na. The final pH was 2.5 and the column temperature was maintained at 25°C, with the flow rate set at 1.0 ml min^{-1} (Okada *et al*., 1995; 1996b; 1997c,d).

Determination of diffusion rates of Ca^{2+} channel blockers

To estimate the rate of ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC diffusion through the membrane, dialysis probes were perfused at a flow rate of 1 μl min^{-1} and placed in the perfusing solution *in vitro*. During analysis the temperature was maintained at 37°C with a perfusion warmer. The amount of ω-conotoxin GVIA, ω-agatoxin IVA and ω-contoxin MVIIC that diffused through the dialysis tube into the extramembrane solution in 120 min was determined by h.p.l.c. The h.p.l.c. system used for determination of the levels of ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC was equipped with a pump (L-4000, Yanaco Co., Japan) and a u.v. detector (UV-970, Jusco Co., Japan) set at 210 nm. The analytical column (Prospher RP-18, 125 mm × 4 mm internal diameter, particle size 5 μm) was purchased from Kanto Kagaku Co. (Japan). The mobile phase was 0.1 M sodium chloride buffer (pH 2,4), containing 10%, 20% and 30% (v/v) acetonitrile for determination of concentrations of ω-conotoxin MVIIC, ω-conotoxin GVIA and ω-agatoxin IVA. The column temperature was maintained at 25°C with the flow rate set at 1.0 ml min^{-1}.

Study design

Experiment 1: effects of Ca^{2+} channel blockers on striatal basal extracellular levels of dopamine and DOPA To study the effects of Ca^{2+} channel blockers on striatal extracellular dopamine and DOPA levels, perfusion with modified Ringer solution was commenced. When the striatal extracellular dopamine and DOPA levels had stabilized (control; Okada *et al*., 1997c) the perfusion medium was switched to modified Ringer solution containing the required agent, either ω-conotoxin GVIA (from 0.01 to 10,000 nM), ω-agatoxin IVA (from 0.01 to 10,000 nM) or ω-conotoxin MVIIC (from 0.01 to 10,000 nM).

Experiment 2: effects of Ca^{2+} channel blockers on Ca^{2+}-evoked striatal extracellular levels of dopamine and DOPA To study the interaction between Ca^{2+} channel blockers and an increase in the extracellular Ca^{2+} level (from 1.2 mM to 3.4 mM) on striatal extracellular dopamine and DOPA levels, following experiment 1 (control), the perfusion medium was switched to modified Ringer solution containing 3.4 mM Ca^{2+} and perfusion was continued for 20 min.

Experiment 3: effects of Ca^{2+} channel blockers on K^+-evoked striatal extracellular levels of dopamine and DOPA To study the interaction between the Ca^{2+} channel blockers and an increase in the extracellular K^+ level (from 2.7 mM to 50.0 mM) on striatal extracellular dopamine and DOPA levels, following experiment 2, after striatal extracellular dopamine and DOPA levels had stabilized (control), the perfusion medium was switched to modified Ringer solution containing 50 mM K^+ and perfusion was continued for 20 min.

Experiment 4: effects of an increase in extracellular levels of Ca^{2+} and K^+ on tyrosine hydroxylase activity in vivo To study the effects of an increase in the extracellular levels of Ca^{2+} and K^+ on tyrosine hydroxylase activity (detected as DOPA accumulation), perfusion with modified Ringer solution containing either 1 or 10 μM NSD1015 was commenced. When the striatal extracellular DOPA level had stabilized (control), the perfusion medium was switched and either modified Ringer solution containing 50 mM K^+ or 3.4 mM Ca^{2+} was perfused for 20 min, or Ca^{2+}-free modified Ringer solution containing 40 mM Mg^{2+} was perfused for 120 min.

Chemical agents

The chemical agents used in this study were: a selective N-type Ca^{2+} channel blocker called ω-conotoxin GVIA (Peptide

Institute Inc., Japan), a P-type Ca^{2+} channel blocker called ω-agatoxin IVA (Peptide Institute Inc.), a Q-type Ca^{2+} channel blocker called ω-conotoxin MVIIC (Peptide Instiute Inc.) and an aromatic amino acid decarboxylase inhibitor called NSD1015 (m-hydroxybenzylhydrazine, Sigma Chemical Co., U.S.A.).

Statistics

The differences between the mean striatal extracellular DOPA levels (striatal DOPA accumulation) under control conditions and during NSD1015 treatment were analysed by a repeated measurements one-way analysis of variance (ANOVA) with a random blocked design, and Dunnett's multiple comparison test. The differences between the mean striatal extracellular dopamine and DOPA levels or striatal DOPA accumulation under control and Ca^{2+}-evoked, K^+-evoked or Ca^{2+}-free conditions were analysed by a repeated measurements two-way ANOVA with a completely random design test, and Tukey's multiple comparison test. The concentration-effect relationships of the Ca^{2+} channel blockers on basal, Ca^{2+}-evoked and K^+-evoked levels of striatal dopamine and DOPA release were analysed by use of logistic concentration-response curves. Differences with $P<0.05$ were considered to be significant.

Results

The levels of dopamine and DOPA in the striatal perfusate were 51.82 ± 6.93 and 11.36 ± 0.67 fmol per sample (in 20 min), respectively. Previous *in vitro* experiments (Okada *et al.*, 1997a,d) have shown that the recovery rate of probes for dopamine and DOPA (external to internal probes) were $19.22\pm3.63\%$ and $17.65\pm4.76\%$, respectively (data not shown). Thus, the estimated basal striatal extracellular levels of dopamine and DOPA were 13.48 ± 2.22 nM and 3.22 ± 0.26 nM, respectively. An increase in the extracellular Ca^{2+} level from 1.2 mM to 3.4 mM increased the levels of striatal dopamine and DOPA release to $172.8\pm20.53\%$ and $145.3\pm11.78\%$, respectively (Figures 1 and 2). In our pilot study, no statistically significant differences between the first and second Ca^{2+}-evoked striatal dopamine and DOPA releases were observed (data not shown). An increase in the extracellular K^+ level from 2.7 mM to 50 mM increased the levels of striatal dopamine and DOPA release to $499.5\pm56.7\%$ and $653.2\pm83.5\%$, respectively (Figures 1 and 2). In addition to these results of pilot studies, after perfusion with lower than 1 μM of these three Ca^{2+} channel antagonists for 120 min, the basal striatal extracellular levels of dopamine and DOPA recovered to more than 95%. However, after perfusion with more than 3 μM of ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC, the basal striatal extracellular levels of dopamine and DOPA recovered to upto 60, 90 and 85%, respectively (data not shown).

Diffusion rate of Ca^{2+} channel antagonists

The rates at which ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC diffused from the dialysis tube were $1.02\pm0.31\%$, $0.68\pm0.29\%$ and $0.92\pm0.44\%$ of the amounts of corresponding Ca^{2+} channel antagonists that were perfused for 120 min (data not shown), respectively. Thus, in the present study, the estimated concentrations of ω-conotoxin

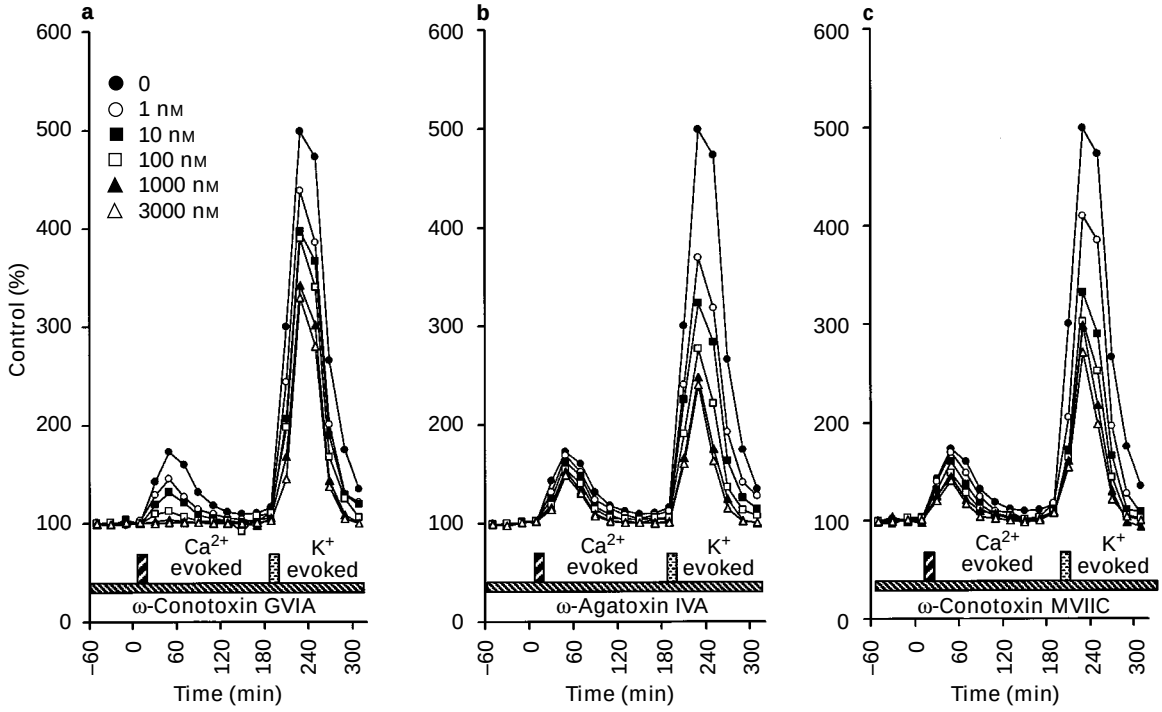

Figure 1 Effects of Ca^{2+} channel antagonists on striatal Ca^{2+}- and K^+-evoked dopamine release. Extracellular dopamine level was measured in striatal perfusate for 60 min during perfusion with 0 (control), 1, 10, 100, 1000 and 3000 nM of Ca^{2+} channel antagonists contained in modified Ringer solution (control). The high Ca^{2+} (3.4 mM: from 0 to 20 min) and high K^+ (50 mM: from 180 to 200 min) containing modified Ringer solution was perfused for 20 min as specified in figures. The effects of an N-type Ca^{2+} channel antagonist, ω-conotoxin GVIA (a), a P-type Ca^{2+} channel antagonist, ω-agatoxin IVA (b) and a Q-type Ca^{2+} channel antagonist, ω-conotoxin MVIIC (c) on striatal Ca^{2+}- and K^+-evoked dopamine release were examined. The ordinate scales indicate the mean ($n=6$) extracellular dopamine level (% control) and the abscissa scales indicate the time in min. Standard deviations were excluded to avoid over complicating the figure.

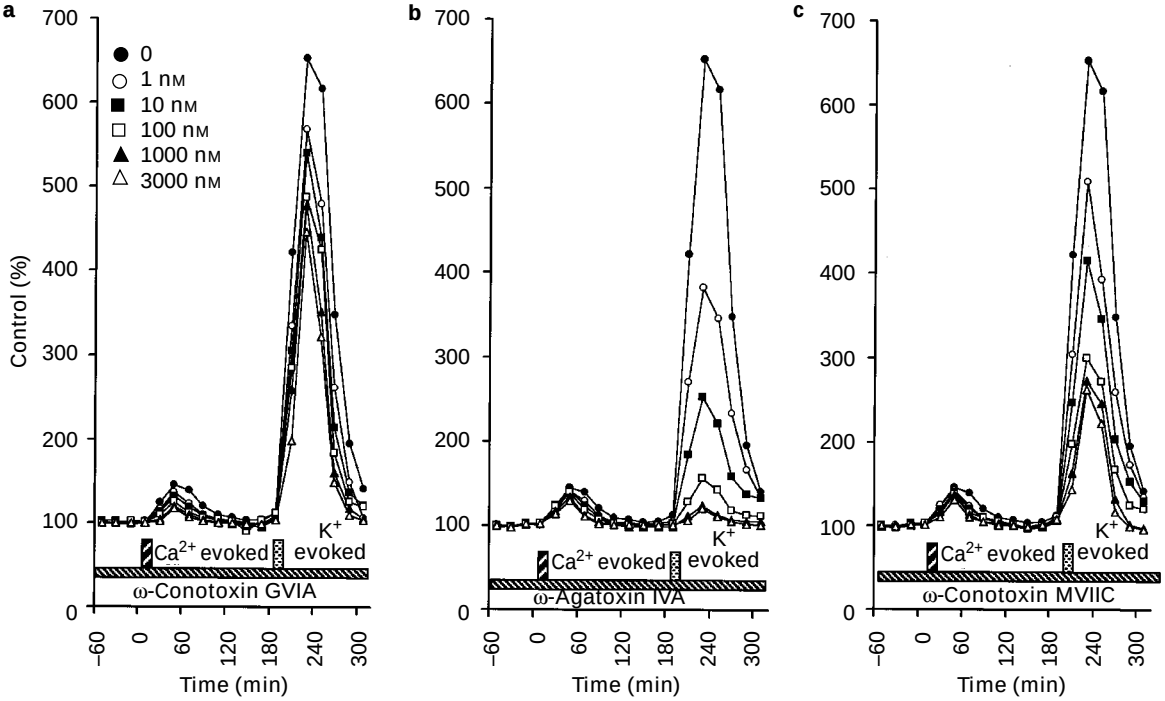

Figure 2 Effects of Ca^{2+} channel antagonists on striatal Ca^{2+}- and K^+-evoked DOPA release. Extracellular DOPA level was measured in striatal perfusate for 60 min during perfusion with 0 (control), 1, 10, 100, 1000 and 3000 nM of Ca^{2+} channel antagonists contained in modified Ringer solution (control), and the high Ca^{2+} (3.4 mM: from 0 to 20 min) and high K^+ (50 mM: from 180 to 200 min) containing modified Ringer solution was perfused for 20 min as specified in figures. The effects of an N-type Ca^{2+} channel antagonist, ω-conotoxin GVIA (a), a P-type Ca^{2+} channel antagonist, ω-agatoxin IVA (b) and a Q-type Ca^{2+} channel antagonist, ω-conotoxin MVIIC (c) on striatal Ca^{2+}- and K^+-evoked DOPA release were examined. The ordinate scales indicate the mean ($n=6$) extracellular DOPA level (% control) and the abscissa scales indicate the time in min. Standard deviations were excluded to avoid over complicating the figure.

GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC in the striatal tissue wee $1.02\times10^{-4}-102$ nM, $0.68\times10^{-4}-68$ nM and $0.92\times10^{-4}-92$ nM, respectively.

Effects of Ca^{2+} channel blockers on basal levels of striatal dopamine and DOPA release

The inhibition of basal levels of striatal dopamine and DOPA release imposed by ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC is shown in Figures 3, 4 and 5. ω-Conotoxin GVIA significantly decreased the basal levels of release of striatal dopamine ($r^2=0.987$, $P<0.01$) and DOPA ($r^2=0.980$, $P<0.01$), in a concentration-dependent manner (Figures 3a, 4a and 5a). The IC_{50} values for ω-conotoxin GVIA inhibition of basal levels of striatal dopamine and DOPA release were 46.9 nM (estimated IC_{50} value was 0.48 nM) and 936.0 nM (estimated IC_{50} value was 9.55 nM), respectively (Figure 5a). Neither ω-agatoxin IVA (Figures 3b, 4b and 5b) nor ω-conotoxin MVIIC (Figures 3c, 4c and 5c) affected the basal levels of release of striatal dopamine and DOPA.

Effects of Ca^{2+} channel blockers on Ca^{2+}-evoked striatal dopamine and DOPA release

The inhibition of Ca^{2+}-evoked striatal dopamine and DOPA release by ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC is shown in Figures 1, 2 and 6. ω-Conotoxin GVIA significantly reduced Ca^{2+}-evoked release of striatal dopamine ($r^2=0.991$, $P<0.01$) and DOPA ($r^2=0.942$, $P<0.05$), in a concentration-dependent manner (Figures 1a, 2a and 6a). The IC_{50} values for ω-conotoxin GVIA inhibition of Ca^{2+}-evoked release of striatal dopamine and DOPA were 39.0 nM (estimated IC_{50} value was 0.40 nM) and 1.03 μM (estimated IC_{50} value was 10.51 nM), respectively (Figure 6a). Neither ω-agatoxin IVA nor ω-conotoxin MVIIC affected the Ca^{2+}-evoked release of striatal dopamine and DOPA (Figures 1b,c; 2b,c; 6b,c).

Effects of Ca^{2+} channel blockers on K^+-evoked striatal dopamine and DOPA release

The inhibition of K^+-evoked striatal dopamine and DOPA release by ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC is shown in Figures 1, 2 and 7. ω-Conotoxin GVIA did not affect the levels of K^+-evoked release of striatal dopamine and DOPA (Figures 1a, 2a and 7a). ω-Agatoxin IVA significantly inhibited the K^+-evoked release of striatal dopamine ($r^2=0.978$, $P<0.01$) and DOPA ($r^2=0.965$, $P<0.01$), in a concentration-dependent manner (Figures 1b, 2b and 7b). The IC_{50} values for ω-agatoxin IVA inhibition of K^+-evoked release of striatal dopamine and DOPA were 389.1 nM (estimated IC_{50} value was 2.65 nM) and 22.2 nM (estimated IC_{50} value was 0.15 nM), respectively (Figure 7b). ω-Conotoxin MVIIC significantly inhibited the K^+-evoked release of striatal dopamine ($r^2=0.950$, $P<0.05$) and DOPA ($r^2=0.997$, $P<0.01$), in a concentration-dependent manner (Figures 1c, 2c and 7c). The IC_{50} values for ω-conotoxin MVIIC inhibition of K^+-evoked release of striatal dopamine and DOPA were 1.36 μM (estimated IC_{50} value was 12.54 nM) and 331.9 nM (estimated IC_{50} value was 3.05 nM), respectively (Figure 7c).

Effects of an increase in extracellular levels of Ca^{2+} and K^{+} on tyrosine hydroxylase activity in vivo

The striatal extracellular DOPA levels had plateaued 120 min after the perfusion medium was switched to modified Ringer solution containing either 1 or 10 μM NSD1015. Perfusion with NSD1015 at both 1 and 10 μM increased extracellular DOPA levels from 3.22 ± 0.26 nM to 379.60 ± 76.49 nM and 1405.10 ± 137.26 nM, respectively (Figure 8a). When striata were perfused without NSD1015, the striatal extracellular DOPA level decreased significantly by 1.48 ± 0.14 nM after the perfusate was changed from modified Ringer solution to Ca^{2+}-free modified Ringer solution ($P<0.01$). However, striata perfused with 1 and 10 μM NSD1015 showed no change in

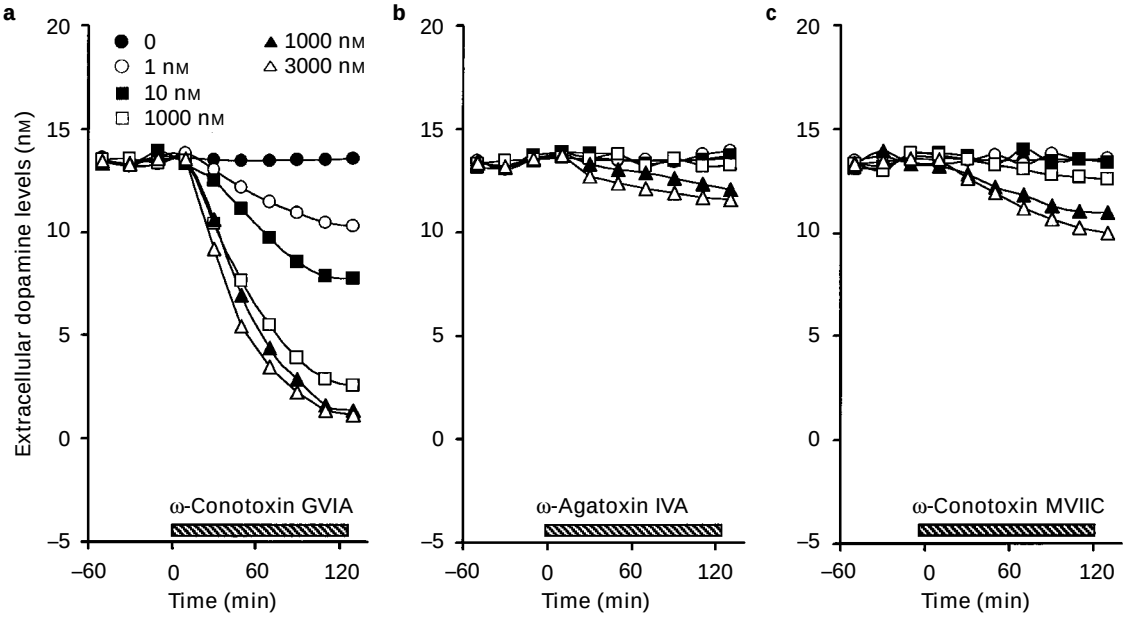

Figure 3 Effects of Ca^{2+} channel antagonists on striatal basal dopamine release. The extracellular dopamine level was measured in striatal perfusate for 60 min during the pre-drug period, and for 120 min during perfusion with modified Ringer's solution containing 0 (control), 1, 10, 100, 1000 and 3000 nM Ca^{2+} channel antagonists. The effects of an N-type Ca^{2+} channel antagonist, ω-conotoxin GVIA (a), a P-type Ca^{2+} channel antagonist, ω-agatoxin IVA (b) and a Q-type Ca^{2+} channel antagonist, ω-conotoxin MVIIC (c) on striatal basal dopamine release were examined. The ordinate scales indicate the mean ($n=6$) estimated extracellular dopamine level (nM) and the abscissa scales indicate the time in min. Standard deviations were excluded to avoid over complicating the figure.

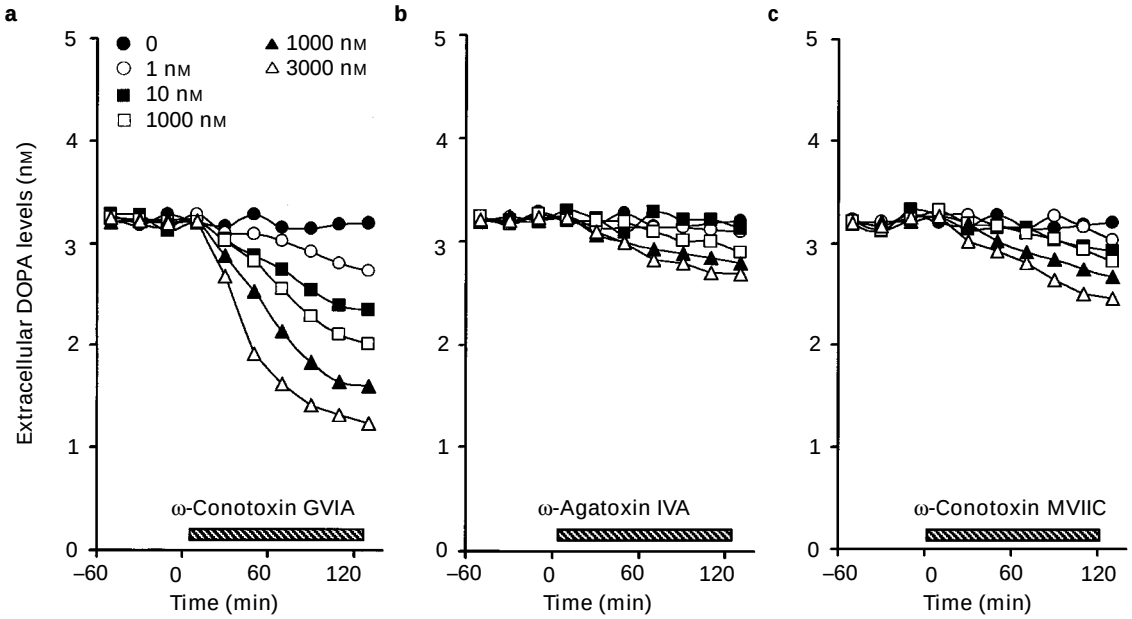

Figure 4 Effects of Ca^{2+} channel antagonists on striatal basal DOPA release. The extracellular DOPA level was measured in striatal perfusate for 60 min during the pre-drug period, and for 120 min during perfusion with modified Ringer solution containing 0 (control), 1, 10, 100, 1000 and 3000 nM Ca^{2+} channel antagonists. The effects of an N-type Ca^{2+} channel antagonist, ω-conotoxin GVIA (a), a P-type Ca^{2+} channel antagonist, ω-agatoxin IVA (b) and a Q-type Ca^{2+} channel antagonist, ω-conotoxin MVIIC (c) on striatal basal DOPA release were examined. The ordinate scales indicate the mean ($n=6$) estimated extracellular DOPA level (nM) and the abscissa scales indicate the time in min. Standard deviations were excluded to avoid over complicating the figure.

striatal extracellular DOPA levels after switching from modified Ringer solution to Ca^{2+}-free modified Ringer solution (Figure 8b). During perfusion with 1 and 10 μM NSD1015, high-Ca^{2+}-evoked stimulation did not affect striatal extracellular DOPA levels (Figure 8c). On the other hand, although NSD1015 significantly ($P<0.01$) inhibited the K^+-

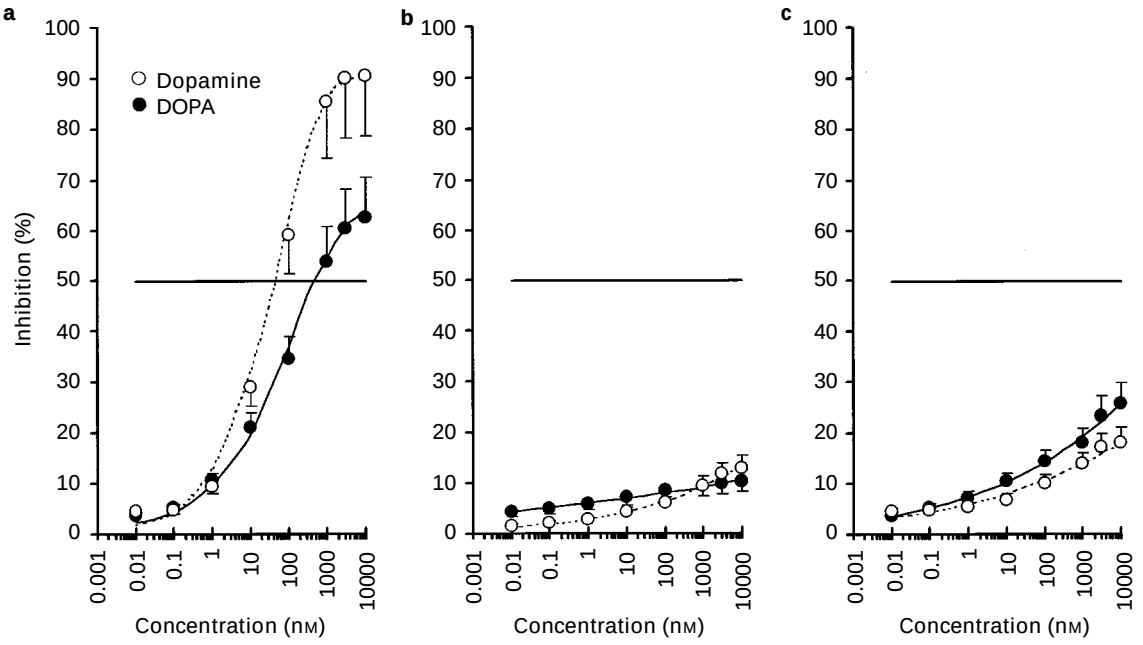

Figure 5 Inhibition of the basal levels of striatal dopamine and DOPA release by ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC. The ordinate scales indicate the basal levels of striatal dopamine ($n=48$) and DOPA ($n=48$) release (nM), which were determined between 100 and 120 min after addition of a Ca^{2+} channel antagonist to the perfusion medium, and the abscissa scales show the logarithmic concentration of the antagonist. The concentration-effect relationships of the Ca^{2+} channel blockers on striatal basal dopamine and DOPA release were analysed by logistic concentration-response curves. ω-Conotoxin GVIA (a) decreased the basal levels of striatal dopamine as well as DOPA release, in a concentration-dependent manner ($P<0.01$). The IC_{50} values for ω-conotoxin GVIA inhibition of the basal levels of striatal dopamine and DOPA release were 0.48 nM and 9.55 nM, respectively. Neither ω-agatoxin IVA (b) nor ω-conotoxin MVIIC (c) affected the basal levels of striatal dopamine or DOPA release.

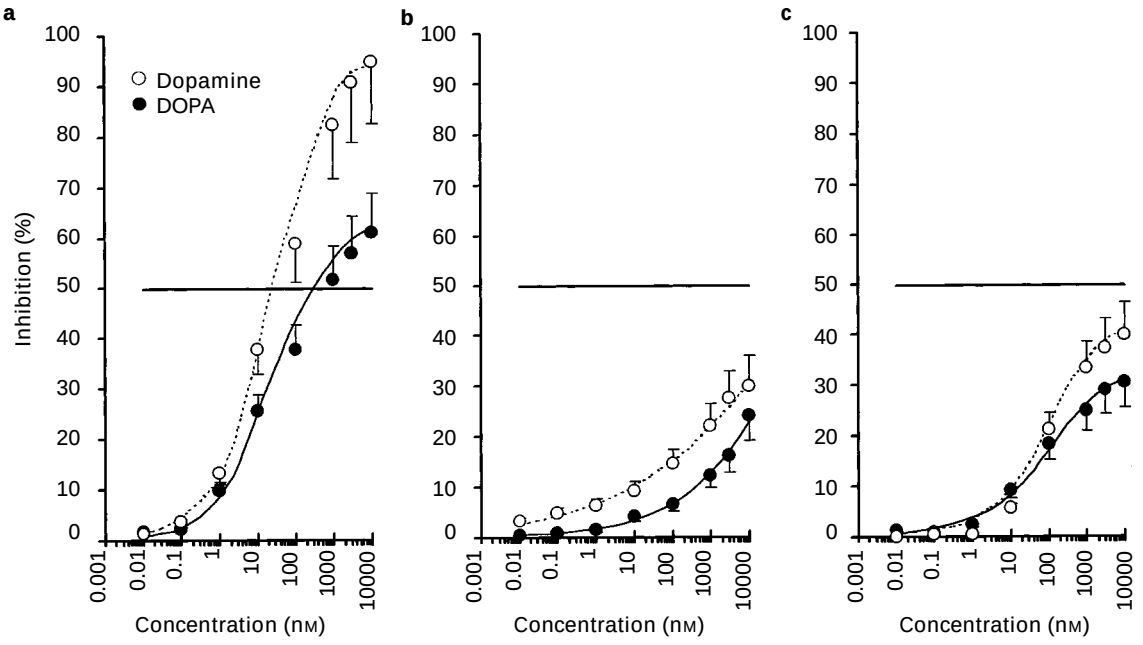

Figure 6 Inhibition of Ca^{2+}-evoked striatal dopamine and DOPA release by ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC. The ordinate scales indicate the percentage of the control value (pre-Ca^{2+} stimulation) of Ca^{2+}-evoked striatal dopamine ($n=48$) and DOPA ($n=48$) release, which were determined between 20 and 40 min after Ca^{2+}-evoked stimulation, and the abscissa scales show the logarithmic concentrations of the antagonist. The concentration-effect relationships of the Ca^{2+} channel blockers on striatal Ca^{2+}-evoked levels of striatal dopamine and DOPA release were analysed by logistic concentration-response curves. ω-Conotoxin GVIA inhibited Ca^{2+}-evoked striatal dopamine and DOPA release, in a concentration-dependent manner ($P<0.01$). The IC_{50} values for ω-conotoxin GVIA (a) inhibition of Ca^{2+}-evoked striatal dopamine and DOPA release were 0.40 nM and 10.51 nM, respectively. Neither ω-agatoxin IVA (b) nor ω-conotoxin MVIIC (c) affected Ca^{2+}-evoked striatal dopamine or DOPA release.

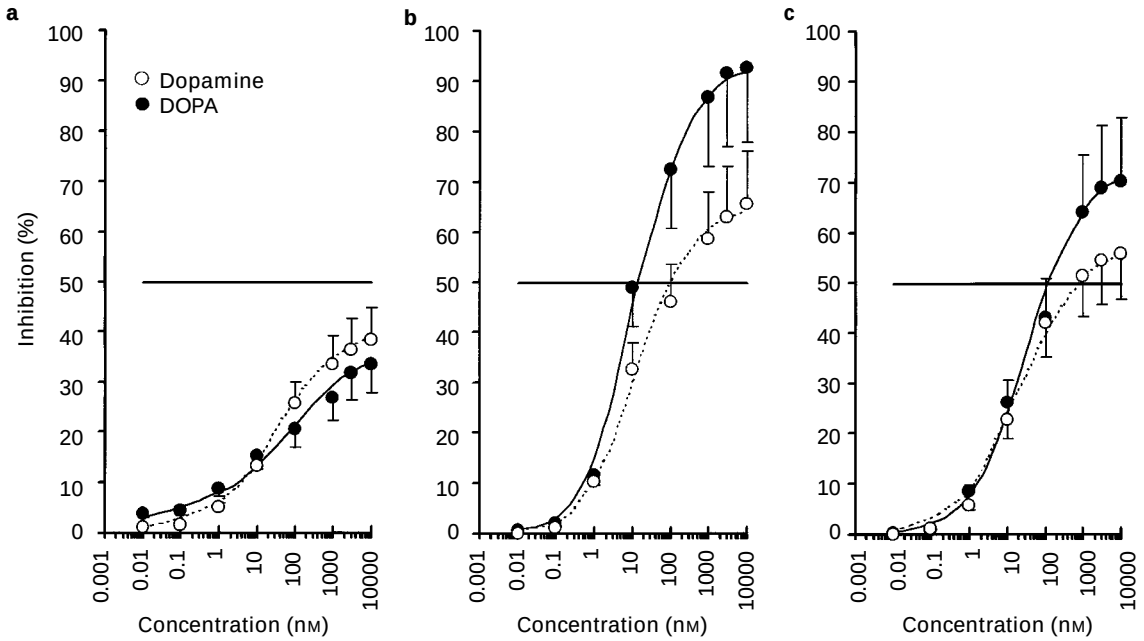

Figure 7 Inhibition of K^+-evoked striatal dopamine and DOPA release by ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC. The ordinate scales indicate the percentage of the control value (pre-K^+ stimulation) of K^+-evoked striatal dopamine ($n=48$) and DOPA ($n=48$) release, which were determined between 20 and 40 min after K^+-evoked stimulation, and the abscissa scales show the logarithmic concentration of the antagonist. The concentration-effect relationships of the Ca^{2+} channel blockers on striatal K^+-evoked level of striatal dopamine and DOPA release were analysed by logistic concentration-response curves. ω-Conotoxin GVIA (a) did not affect K^+-evoked striatal dopamine or DOPA release. ω-Agatoxin IVA (b) inhibited K^+-evoked striatal dopamine as well as DOPA release, in a concentration-dependent manner ($P<0.01$). The IC_{50} values for ω-agatoxin IVA inhibition of K^+-evoked striatal dopamine and DOPA release were 2.65 nM and 0.15 nM, respectively. ω-Conotoxin MVIIC (c) inhibited $K<^+$-evoked striatal dopamine as well as DOPA release, in a concentration-dependent manner ($P<0.01$). The IC_{50} values for ω-conotoxin MVIIC inhibition of K^+-evoked striatal dopamine and DOPA release were 12.54 nM and 3.05 nM, respectively.

evoked elevation of extracellular DOPA levels in a concentration-dependent manner, high-K^+-evoked stimulation significantly increased striatal extracellular DOPA levels under conditions that were unaffected by whether or not NSD1015 was perfused (Figure 8d). Thus, neither Ca^{2+}- nor K^+-evoked stimulation affected DOPA accumulation, which is an index of tyrosine hydroxylase activity *in vivo*.

Discussion

Increases in the extracellular concentrations of Ca^{2+} and K^+ resulted in increased neurotransmitter releases (Okada *et al*., 1992; 1996a; Keith *et al*., 1993), although the degree of the response was different for each transmitter. This is possibly due to differences in the sensitivities of intracellular release mechanisms to changes in internal Ca^{2+} concentration, or to the different proportions of Ca^{2+} channel subtypes present on the nerve terminals (Harvey *et al*., 1996).

Recently, a Ca^{2+}-dependent, tetrodotoxin (TTX)- and K^+-sensitive release of striatal DOPA has been described in detail by Misu and his colleagues (Misu & Goshima, 1993; Misu *et al*., 1995a,b). The present study also demonstrated Ca^{2+}-dependent and K^+-sensitive release of striatal DOPA, since perfusion with Ca^{2+}-free modified Ringer solution and K^+-evoked stimulation decreased and increased the extracellular levels of striatal DOPA, respectively. However, perfusion with Ca^{2+}-free and 40 nM Mg^{2+}-containing modified Ringer solution caused the extracellular levels of striatal dopamine to drop drastically to less than 10% of basal dopamine levels (data not shown); those of striatal DOPA were weakly reduced to 40% of basal levels. In order to determine whether the elevation of striatal extracellular levels of DOPA caused by perfusion with high concentrations of Ca^{2+} (3.4 mM) and K^+ (50 mM) is influenced by the tyrosine hydroxylase activity evoked by high concentrations of Ca^{2+} and K^+ (Nestler & Greengard, 1994), we examined DOPA accumulation in the striatal perfusate in the presence of NSD1015, which can be used as an index of *in vivo* tyrosine hydroxylase activity (Westerink *et al*., 1990; Okada *et al*., 1995; 1997b). The striatal extracellular DOPA levels were affected by increases or decreases in extracellular Ca^{2+} or K^+ levels in the absence of NSD1015. However, in the presence of NSD1015 the level of striatal DOPA accumulation was insensitive to changes in the extracellular Ca^{2+} level. The level of striatal DOPA accumulation increased during high-K^+-evoked stimulation, but the sensitivity of striatal extracellular DOPA levels to high-K^+-evoked stimulation was reduced by NSD1015, in a concentration-dependent manner ($P<0.01$). Thus, the elevation of striatal extracellular dopamine and DOPA levels induced by either high-Ca^{2+}- or high-K^+-evoked stimulation may not depend upon tyrosine hydroxylase activity. In addition, the present results suggest that there are two sources of striatal extracellular DOPA: that released in response to stimulation, and the metabolic basal flow of DOPA, since striatal extracellular DOPA levels were greater than 1 nM, even during perfusion with Ca^{2+}-free modified Ringer solution containing 40 mM Mg^{2+} (which prevents neurotransmitter release from nerve terminals; Augustine *et al*., 1987; Westerink *et al*., 1988; 1989; Okada *et al*., 1996a).

The findings of the study described here suggest that the basal level of striatal dopamine and DOPA release is regulated mainly by N-type Ca^{2+} channels, but not by P- nor Q-type Ca^{2+} channels.

The mechanism of striatal dopamine and DOPA release include a Ca^{2+}-sensitive pathway, since an increase in the

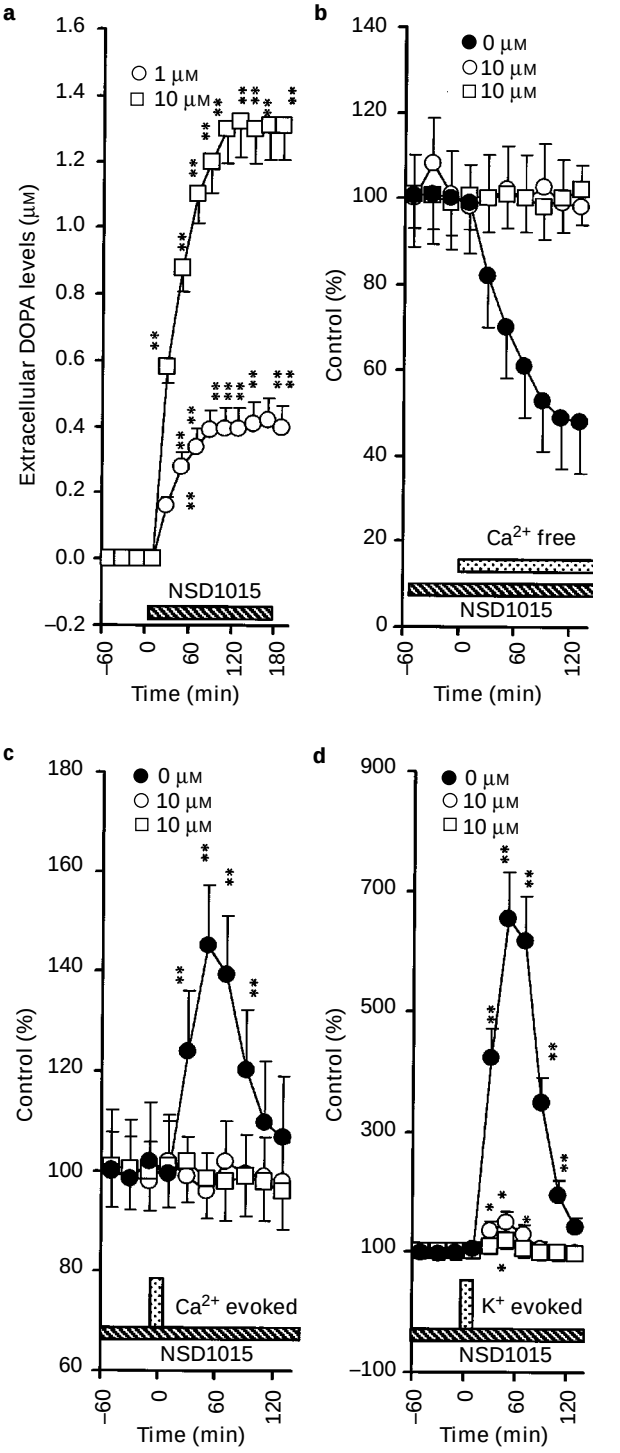

Figure 8 Effects of an increase in extracellular levels of Ca^{2+} and K^+ on tyrosine hydroxylase activity *in vivo*. (a) The effects of 1 and 10 μM NSD1015 on striatal extracellular DOPA levels (DOPA accumulation). The extracellular DOPA level was measured in striatal perfusate for 60 min before the addition of NSD1015 (control) and for 180 min during perfusion with NSD1015. The ordinates indicate the mean, and vertical lines s.e. ($n=6$), estimated extracellular DOPA level (μM) and the abscissa scale indicates the time in min. The mean values obtained before and during perfusion with NSD1015 were compared by repeated measurements one-way analysis of variance with a random blocked design and Dunnett's multiple comparison test (*$P<0.05$; **$P<0.01$). (b) The effects of Ca^{2+}-free modified Ringer solution containing 40 mM Mg^{2+} on striatal DOPA accumulation, in the presence of 0, 1 or 10 μM NSD1015. The ordinates indicate the mean ($n=6$) of extracellular DOPA level (% control) and the abscissa scale indicates the time in min. The mean values obtained before and during perfusion with Ca^{2+}-free modified Ringer solution containing 40 mM Mg^{2+} were compared by repeated measurements one-way analysis of variance with a random blocked design and Dunnett's multiple comparison test (*$P<0.05$; **$P<0.01$). The effects of Ca^{2+}- (c) and K^+-evoked (d) stimulation on striatal DOPA accumulation, in the presence of 0, 1 or 10 μM NSD1015 were examined ($n=6$). The mean values obtained before and after Ca^{2+} and K^+ stimulation were compared by repeated measurements one-way analysis of variance with a random blocked design and Dunnett's multiple comparison test (*$P<0.05$; **$P<0.01$).

extracellular Ca^{2+} level from 1.2 mM to 3.4 mM resulted in increased dopamine and DOPA release to $172.8\pm20.53\%$ and $145.3\pm11.78\%$, respectively. The Ca^{2+}-evoked striatal release of dopamine and DOPA was regulated mainly by N-type Ca^{2+} channels, whereas P- and Q-type Ca^{2+} channels did not affect Ca^{2+}-sensitive dopamine or DOPA release. Thus, by use of an *in vivo* microdialysis preparation it was substantiated that the Ca^{2+}-evoked release of striatal dopamine and DOPA is mediated by N-type Ca^{2+} channel function. In addition, striatal dopamine release was shown to be more sensitive than striatal DOPA release to N-type Ca^{2+} channels, since the IC_{50} value of ω-conotoxin GVIA for striatal dopamine release was lower than that for striatal DOPA release.

It has been well established that ω-conotoxin GVIA, ω-agatoxin IVA and ω-conotoxin MVIIC are irreversible toxic Ca^{2+} channel antagonists (Randall & Tsien, 1995). In our pilot study, the effects of more than 3 μM of these three Ca^{2+} channel antagonists on basal striatal extracellular levels of dopamine and DOPA were irreversible, indicating the toxic effects of these three Ca^{2+} channel antagonists. The effects of less than 1 μM of these three Ca^{2+} channel antagonists on basal striatal extracellular levels of dopamine and DOPA are probably due to their Ca^{2+} channel antagonistic activity rather than irreversible toxic effects, since the inhibitory effects of reversible concentrations of these Ca^{2+} channel antagonists (lower than 1 μM) were concentration-dependent, whereas from 1 μM (reversible concentration) to 10 μM (irreversible concentration), the inhibitory effects of these three Ca^{2+} channel antagonists reached a plateau.

The mechanism of striatal dopamine and DOPA release also involve a K^+-sensitive pathway, since an increase in the extracellular K^+ level from 2.7 mM to 50 mM increased dopamine and DOPA release to $499.5\pm56.7\%$ and $653.2\pm83.5\%$, respectively. The K^+-evoked release of both striatal dopamine and DOPA was inhibited by P- and Q-type Ca^{2+} channel antagonists, whereas the N-type Ca^{2+} channel antagonists did not affect the K^+-evoked release of either striatal dopamine or DOPA. In particular, ω-agatoxin IVA drastically inhibited K^+-evoked striatal DOPA release. The sensitivities of P- and Q-type Ca^{2+} channels to K^+-evoked DOPA release were higher than those to K^+-evoked dopamine release.

ω-Agatoxin IVA has been shown to block the P-type Ca^{2+} channel with an IC_{50} of about 1 nM (Randall & Tsien, 1995), and it has been proposed that a higher concentration (more than 90 nM) blocks the Q-type Ca^{2+} channel present in granule cells (Randall & Tsien, 1995). ω-Conotoxin MVIIC has been shown to block both P- and Q-type Ca^{2+} channels at concentrations of no more than 500 nM (Randall & Tsien, 1995). In addition, this toxin also weakly inhibits the N-type Ca^{2+} channel (Hillyard *et al*., 1992). However, in the present study, the estimated concentrations of ω-conotoxin MVIIC and ω-agatoxin IVA were lower than 100 nM. Therefore, the present study demonstrated the selective effects of each Ca^{2+} channel antagonist on basal, and Ca^{2+}- and K^+-evoked striatal dopamine and DOPA release.

Recently, the question has been raised as to which of the Ca^{2+} channel subtypes is predominantly associated with neurotransmission. In the cerebellum, 21% of the GABAergic Purkinje cells have been shown to be ω-conotoxin-GVIA-sensitive, and 73% to be ω-agatoxin-IVA-sensitive (Takahashi & Momiyama, 1993). In region CA1 of the hippocampus, which is a glutamatergic-neurone-rich area, the rates of occurrence of ω-conotoxin-GVIA- and ω-agatoxin-IVA-sensitive neurones were shown to be 12% and 46%, respectively (Takahashi & Momiyama, 1993). The present results indicate that in the striatum, P- and Q-type Ca^{2+}-channel-sensitive DOPA transmission is more dominant than N-type Ca^{2+}-channel-sensitive DOPA transmission. Therefore, the pattern of striatal DOPA transmission may be intermediate between that of striatal dopamine and hippocampal glutamate transmission. To elucidate this a further study, in which the effects of N-, P- and Q-type Ca^{2+} channel subtypes on hippocampal glutamate transmission is investigated with *in vivo* microdialysis, is required.

Both N- and P-type Ca^{2+}-channel-insensitive hippocampal excitatory synaptic transmission are completely blocked by ω-conotoxin MVIIC (Wheeler *et al*., 1994). However, in the present study we were unable to demonstrate the characteristic function of striatal Q-type Ca^{2+} channels, since ω-conotoxin MVIIC exhibited an effect which was similar to that of ω-agatoxin IVA on basal as well as Ca^{2+}- and K^+-evoked striatal dopamine and DOPA release. Dunlap *et al*. (1995) have suggested that differences between the functions of P- and Q-type Ca^{2+} channels cannot be distinguished, since both channels possess the α_{1A} subunit. These observations suggest that the actions of both the P- and Q-type Ca^{2+} channel subtypes on the striatal transmission of dopamine and DOPA are similar (Dunlap *et al*., 1995).

The transmitter-like function of DOPA has not been considered to be clinically important. However, the effects of L-DOPA, which is the metabotropic precursor of dopamine, have received much attention since its administration is frequently used in the treatment of Parkinson's disease and neuroleptic malignant syndrome, both of which are the result of dopaminergic dysfunction (Misu *et al*., 1995a,b; Otani *et al*., 1991a,b). We have already found the efficacy of L-DOPA administration for neuroleptic malignant syndrome. However, it is not clear whether administration of L-DOPA or dopamine receptor agonists are more effective for the treatment of neuroleptic malignant syndrome. On the other hand, in patients with Parkinson's disease who are on long-term L-DOPA treatment, 'on-off' and 'wearing-off' of the effects of L-DOPA occur frequently (Misu *et al*., 1995a,b). Conversely, in similar long-term dopamine receptor agonist treatent, these side-effects occur only rarely (Goetz *et al*., 1983). Further specific clinical trials are required to improve L-DOPA therapy for the treatment of Parkinson's disease or neuroleptic malignant syndrome.

We conclude that both the basal release and Ca^{2+}-evoked release of striatal DOPA are weakly regulated by N-type Ca^{2+} channels, whereas both P- and Q-type Ca^{2+} channels strongly regulate K^+-evoked striatal DOPA release. Both the basal release and Ca^{2+}-evoked release of striatal dopamine are strongly regulated by N-type Ca^{2+} channels, whereas both P- and Q-type Ca^{2+} channels weakly regulate K^+-evoked striatal dopamine release. The sensitivity of striatal basal and Ca^{2+}-evoked DOPA transmission to N-type Ca^{2+} channels is lower than that of striatal basal and Ca^{2+}-evoked dopamine transmission. However, the sensitivity of striatal K^+-evoked DOPA transmission to P/Q-type Ca^{2+} channels is higher than that of striatal K^+-evoked dopamine transmission. These findings suggest that striatal DOPA transmission is neurotransmitter-like and, unlike the mechanisms of striatal dopaminergic transmission, is partly regulated by voltage-sensitive Ca^{2+} channels.

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