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D_2-dopamine receptor-mediated inhibition of intracellular Ca^{2+} mobilization and release of acetylcholine from guinea-pig neostriatal slices

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1 The effect of dopamine receptor activation on electrically- or high K^+ (30 mM)-evoked neurotransmitter release and rise in intracellular Ca^{2+} concentration was investigated using slices of guinea-pig neostriatum.

2 A specific D_2-dopamine receptor agonist, LY-171555 (a laevorotatory enantiomer of LY-141865: N-propyl tricyclic pyrazole) at 10^{-6} M inhibited electrical stimulation- and high K^+-evoked release of [^{3}H]-acetylcholine ([^{3}H]-ACh) to 47.7 ± 6.0% and 54.1 ± 5.0% of control, respectively. The maximal inhibition by LY-171555 at 10^{-5} M was 54.8 ± 5.1% reduction of the control. The half-maximal effective concentration (EC_{50}) of LY-171555 for the inhibition of [^{3}H]-ACh release was 2.3×10^{-7} M. A specific D_2-dopamine receptor antagonist, (−)-sulpiride (10^{-7} M) reversed the inhibition of [^{3}H]-ACh release induced by LY-171555. A specific D_1-dopamine receptor agonist, SK&F 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-benzazepine) (10^{-5} M) had no effect on the release of [^{3}H]-ACh. LY-171555 (10^{-6} M) also inhibited the high K^+-evoked endogenous glutamate release, by 47% of control. This inhibitory effect was reversed by (−)-sulpiride (10^{-7} M).

3 We used a fluorescent, highly selective Ca^{2+} indicator, 'quin 2' to measure intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). Electrical stimulation of slices preloaded with quin 2 led to an elevation of relative fluorescence intensity and this response was reduced by the removal of Ca^{2+} from the bathing medium. These results indicate that the enhanced elevation in fluorescence intensity in the quin 2-loaded slices reflects the increase of intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$.

4 The mixed D_1- and D_2-receptor agonist, apomorphine and LY-171555 inhibited the increase of $[Ca^{2+}]_i$ induced by electrical stimulation or high K^+ medium, in a concentration-dependent manner, while SK&F 38393 did not affect the increase of $[Ca^{2+}]_i$. The maximal inhibitory effect of LY-171555 at 3×10^{-5} M was 35 ± 3% reduction in control values. The inhibitory effect of LY-171555 was antagonized by (−)-sulpiride (10^{-7} M).

5 There was a high correlation ($r = 0.997$, $P < 0.05$) between the D_2-receptor-mediated inhibition of the stimulated rise of $[Ca^{2+}]_i$ and [^{3}H]-ACh release.

6 When the slices were superfused with the Ca^{2+}-free medium containing EGTA (10^{-4} M) for 5 min, the rise in $[Ca^{2+}]_i$ was markedly suppressed to 18.0% of control by LY-171555 (10^{-6} M).

7 These data indicate that activation of the D_2-dopamine receptor suppresses the elevation of $[Ca^{2+}]_i$ induced by depolarizing stimuli. This may be due to inhibition of mobilization of Ca^{2+} from the intracellullar store. We propose that the D_2-receptor-mediated inhibition of transmitter release is probably due to a reduction in intracellular Ca^{2+} mobilization.

Introduction

The existence of presynaptic D_2-dopamine receptors mediating inhibitory control is well documented with regard to the release of acetylcholine (ACh) from nerve terminals of striatal cholinergic interneurones (Stoof & Kebabian, 1982; Scatton, 1982; Cubeddu & Hoffmann, 1983; Baud *et al.*, 1985), of dopamine from nigrostriatal dopaminergic nerve terminals (Arbilla & Langer, 1981; Cubeddu & Hoffmann, 1982; Lehmann *et al.*, 1983) and of glutamate (Glu) from cortico-striatal glutamatergic nerve terminals (Mitchell &

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Dogget, 1980; Rowlands & Roberts, 1980). It is possible that the inhibition of neurotransmitter release mediated by D_2-dopamine receptors involves the prevention of the availability of Ca^{2+} for Ca^{2+}-linked neurotransmitter release mechanisms. The inhibition of mechanisms related to the release of neurotransmitters, as regards those mediated by the D_2-dopamine receptor is not well understood.

Tsien *et al.* (1982a,b) developed the fluorescent quinoline Ca^{2+} indicator, 'quin 2', which has a high affinity for Ca^{2+}, very low affinity for Mg^{2+} and H^+, and exhibits large absorbance and fluorescence changes resulting from Ca^{2+}-binding. Quin 2 trapped inside intact cells by means of nonpolar ester derivatives (quin 2-acetoxymethyl ester: quin 2/AM) crosses the plasma membrane and is hydrolyzed intracellularly back to the parent membrane-impermeable polycarboxylate anions. By continuously monitoring intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) changes in neostriatal slices, we found that activation of the D_2-dopamine receptor inhibited depolarizing stimuli-evoked intracellular mobilization of Ca^{2+} and that presynaptic inhibition by D_2-dopamine receptor activation is due to its inhibitory action on Ca^{2+} mobilization.

Methods

[^{3}H]-acetylcholine release and endogenous glutamate release

Guinea-pigs of either sex (250–300 g) were decapitated, the brain quickly removed and cut coronally at the level of the optic chiasma and rostal pole of the neostriatum. The striatal slices were dissected with a razor blade and a sliding guide. The caudal part of the striatum and globus pallidus was excluded. Frontal sections used in the experiments were 350–450 μm thick and weighed 6–8 mg. Slices of the guinea-pig neostriatum were preloaded with [^{3}H]-choline (10^{-7} M) for 1 h at 37°C gassed with 95% O_2 and 5% CO_2 and then rinsed with fresh Krebs-Ringer bicarbonate (KRB) solution. The normal KRB solution consisted of (mM) NaCl 118, KCl 3.0, $CaCl_2$ 2.0, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 23 and glucose 11. The preparation was impaled with a pair of parallel platinum electrodes (0.2 mm in diameter, distance 0.7 mm, length 1.5 mm), mounted in the superfusion chamber and then superfused at a constant flow rate of 0.2 ml min^{-1} with KRB solution oxygenated with 95% O_2 and 5% CO_2. The superfusate was continuously collected every 5 min and the radioactivity determined in a liquid scintillation spectrometer (Beckman, model LS-7000). Electrical stimulation (current 1 mA, frequency 1 Hz, pulse duration 1 ms) was applied for 2 min. Unchanged [^{3}H]-ACh in the superfusate was determined as follows: superfusates from electrically stimulated slices, before and during stimulation, were collected for 5 min. Electrical stimulation (1 mA, 1 Hz, 1 ms) was applied for 2 min at 15 and 40 min after the start of superfusion. The superfusates were collected in 1 ml of 3-heptanone-tetraphenylboron (10 mg ml^{-1}) on ice. [^{3}H]-ACh and [^{3}H]-choline were extracted with HCl, dried, dissolved in 1 N formic acid-acetone (15:85, v/v), and separated by an electrophoretic method (Saijoh *et al.*, 1985). The radioactive compounds recovered on dried paper strips were extracted with 0.5 ml ethanol, and the radioactivity measured in a toluene base scintillator, using a liquid scintillation spectrometer (Beckman, model LS-7000). The amounts of [^{3}H]-ACh in the sample obtained before and during stimulation were 72 ± 8 and 81 ± 6% of the total radioactivity, respectively. The total radioactivity in the superfusate was considered to approximate the amount of [^{3}H]-ACh and therefore will be denoted as [^{3}H]-ACh release. The slices were stimulated by superfusing with high K^+ (30 mM) medium which was prepared by equimolar replacement of NaCl by KCl. The evoked release induced by electrical stimulation or high K^+ medium was represented as the 'fold' increase over spontaneous release, which was calculated by dividing the difference between the stimulated release and the spontaneous release by the spontaneous release.

Endogenous glutamate (Glu) release experiments were performed, using the following procedure. The neostriatal slices were placed in a superfusion chamber and superfused at a constant flow rate of 0.2 ml min^{-1} with oxygenated KRB solution. The superfusate was continuously collected every 5 min and the Glu released to the superfusate was extracted by the method of Iversen *et al.* (1971). The samples were passed through a column of Dowex-50W × 8 resin (100 to 200 mesh, H^+ form). The columns were rinsed until the eluate was at neutral pH, and Glu was eluted with 1.5 ml of 2 M NH_4OH. The eluates were evaporated *in vacuo*, and the residues dissolved in 200 μl of distilled water. The amount of Glu was measured by a sensitive enzymatic fluorometric method (Nitsch *et al.*, 1979).

Measurement of intracellular Ca^{2+} level

Using the method of Tsien *et al.* (1982a,b), the slices were loaded by incubation with 1 mM quin2/AM and 0.05% (v/v) dimethylsulphoxide in Ca^{2+}-free KRB solution gassed with 95% O_2 and 5% CO_2 at 37°C for 30 min. Following a further 50 fold dilution with warm KRB solution, incubation was continued for 30 min to allow for completion of the intracellular hydrolysis to quin 2 while encouraging the acetic acid and formaldehyde also formed to diffuse out (Ashley *et al.*, 1984). The preparation was washed 3 times with normal

KRB solution. As described previously (Saijoh *et al.*, 1985; Tanaka *et al.*, 1986), fluorescence was measured using the following procedure. The slices preloaded with quin2/AM were pinned to a thin plate of silicon rubber with a pair of parallel platinum electrodes (0.2 mm in diameter, distance 1.5 mm length 3 mm) and then mounted diagonally in a square quartz cuvette. The slices were perfused at a constant rate of 1 ml min^{-1} with oxygenated KRB solution maintained at 37°C. Fluorescence in the quin2-loaded slices was measured using an Aminco-Bowman spectrofluorometer. The excitation and emission wave lengths were 339 nm with bandpass 4 nm slits and 492 nm with bandpass 10 nm slits, respectively. The slices were stimulated by electrical monophasic rectangular pulses (2.0 mA, 2.5 ms, 5 Hz and train of 25 pulses) or by high K^+ (30 mM) medium, prepared by an equimolar replacement of NaCl with KCl.

Statistical analysis

Data were analysed by Student's *t* test and differences were considered to be significant at the 0.05 level of probability.

Drugs and chemicals

Drugs and chemicals used included: [^{3}H]-choline (80 Ci $mmol^{-1}$, New England Nuclear), quin2/AM (Dojin, Japan), tetrodotoxin (TTX; Sankyo), LY-171555 (a laevorotatory enantiomer of LY-141865: N-propyl tricyclic pyrazole, Eli Lilly), apomorphine (Sigma), SK&F 38393 (2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1H-3-benzazepine, Smith Kline & French), (−)-sulpiride (Fujisawa). All other chemicals were of reagent grade.

Results

Effect of dopamine receptor agonists and an antagonist on [^{3}H]-acetylcholine release and endogenous glutamate release

The electrical stimulation of neostriatal slices preloaded with [^{3}H]-choline produced an increase in tritium efflux. This efflux was inhibited when the slices were superfused with medium containing 10^{-7} M tetrodotoxin (TTX) for 3 min or Ca^{2+}-free KRB solution for 15 min, as already demonstrated (Saijoh *et al.*, 1985). A specific D_2-dopamine receptor agonist, LY-171555 inhibited, in a concentration-dependent manner, the electrically stimulated release of [^{3}H]-ACh, with no change in the spontaneous release (Figure 1). The maximal inhibitory effect of LY-171555 at 10^{-5} M was a 55% reduction of the control values. LY-171555 was half-maximally effective at a concentration of 2.3×10^{-7} M. Apomorphine (10^{-6} M) also inhibited the stimulated [^{3}H]-ACh release, by 52 ± 8.2% of control value. A specific D_2-dopamine receptor antagonist, (−)-sulpiride (10^{-7} M) reversed the inhibition of [^{3}H]-ACh release induced by LY-171555 (10^{-6} M). A specific D_1-dopamine receptor agonist, SK&F 38393 (10^{-5} M) did not affect the stimulated [^{3}H]-ACh release nor the inhibition of [^{3}H]-ACh release induced by LY-171555 (Table 1).

[^{3}H]-ACh release was induced by the application of high K^+ (30 mM) medium. The evoked release of [^{3}H]-

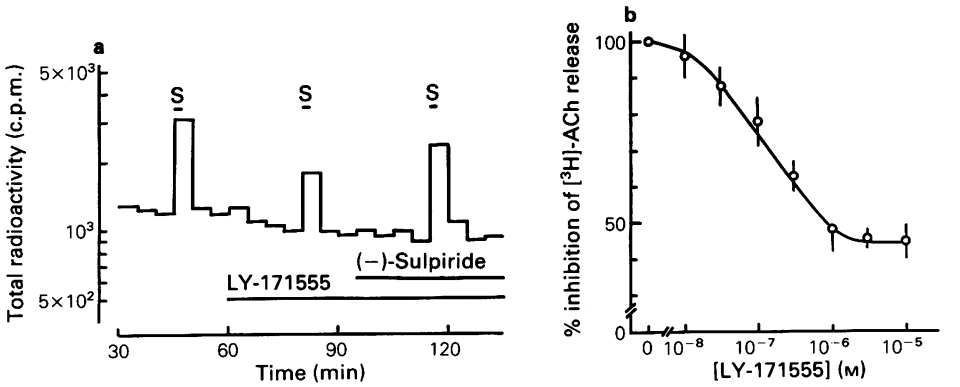

Figure 1 (a) Effect of LY-171555 (10^{-6} M) and (−)-sulpiride (10^{-7} M) on electrically stimulated [^{3}H]-acetylcholine ([^{3}H]-ACh) release from neostriatal slices preloaded with [^{3}H]-choline. S, indicates electrical stimulation (1 mA, 1 Hz, 1 ms for 2 min). (b) Shows the concentration-dependent inhibitory effect of LY-171555 on [^{3}H]-ACh release. Each point is mean from 4 determinations expressed as % of the stimulated release of [^{3}H]-ACh in control medium; vertical lines indicate s.e.mean.

Table 1 Effect of various dopamine receptor agonists and an antagonist on [^{3}H]-acetylcholine ([^{3}H]-ACh) release from neostriatal slices

Treatment	*Evoked release of [^{3}H]-ACh*	*% of control*	*Relative fluorescence intensity* (arbitary units)	*% of control*
(A) Electrical stimulation-induced release				
None	1.68 ± 0.24 ($n = 4$)		37.8 ± 4.1 ($n = 4$)	
LY-171555 (10^{-6} M)	0.87 ± 0.18* ($n = 4$)	47.7	29.1 ± 2.3* ($n = 4$)	77.0
Apomorphine (10^{-6} M)	0.80 ± 0.16* ($n = 4$)	52.0	27.2 ± 4.3* ($n = 4$)	72.0
LY-171555 (10^{-6} M) + (−)-sulpiride (10^{-7} M)	1.59 ± 0.28	94.5	34.5 ± 3.1 ($n = 4$)	91.3
SK&F 38393 (10^{-5} M)	1.66 ± 0.31	98.6	37.1 ± 4.8 ($n = 4$)	98.2
None	1.82 ± 0.19 ($n = 4$)		45.5 ± 3.9 ($n = 5$)	
Ca^{2+}-free medium‡	0.65 ± 0.13* ($n = 4$)	35.7	21.0 ± 2.7* ($n = 5$)	46.1
Ca^{2+}-free medium‡ + LY-171555 (10^{-6} M)	0.21 ± 0.07** ($n = 4$)	12.0	8.19 ± 1.2** ($n = 5$)	18.0
(B) High K^+-induced release				
None	2.61 ± 0.52		36.3 ± 4.4 ($n = 4$)	
LY-171555 (10^{-6} M)	1.40 ± 0.35* ($n = 4$)	54.1	20.2 ± 3.2* ($n = 4$)	56.5
LY-171555 (10^{-6} M) + (−)-sulpiride (10^{-7} M)	2.40 ± 0.43 ($n = 4$)	92.1	32.1 ± 5.8 ($n = 4$)	88.4

[^{3}H]-Acetylcholine release from neostriatal slices preloaded with [^{3}H]-choline was induced by electrical stimulation (1 mA, 1 Hz, 1 ms for 2 min) or perfusion with high K^+ (30 mM) medium for 30 s. In experiments to determine the increase of $[Ca^{2+}]_i$, stimulation was performed at 2 mA, 5 Hz, 2.5 ms for 5 s. Slices were also stimulated by application of high K^+ (30 mM) medium for 30 s. ‡Slices were superfused with Ca^{2+}-free Krebs-Ringer bicarbonate (KRB) solution containing 10^{-4} M EGTA for 5 min. *Significantly different from each control ($P < 0.05$). **Significantly different from value in Ca^{2+}-free medium ($P < 0.05$).

ACh induced by high K^+ (30 mM) medium in the control was 2.61 ± 0.52 ($n = 4$). Whereas the evoked release of [^{3}H]-ACh in the presence of LY-171555 (10^{-6} M) and LY-171555 plus (−)-sulpiride (10^{-7} M) was 1.40 ± 0.35 ($n = 4$) (significantly different from control, $P < 0.05$) and 2.40 ± 0.43 ($n = 4$), respectively. Hence LY-171555 (10^{-6} M) reduced high K^+ medium-induced [^{3}H]-ACh release to 54.1% of the control (Table 1).

To confirm the specificity of LY-171555-induced inhibition of neurotransmitter release, the effect of LY-171555 on the release of endogenous Glu, another striatal neurotransmitter, was also examined. The spontaneous release of Glu slightly declined 67.8 ± 8.5 to 61 ± 8.1 pmol mg^{-1} wet weight 5 min^{-1}, but this was not significant. Superfusion of the slices with a KRB solution containing K^+ (30 mM) evoked an increase of Glu release which was completely inhibited in the Ca^{2+}-free KRB solution containing ethyleneglycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA, 10^{-4} M). These results indicate that the released Glu is of neuronal origin. When slices were superfused with KRB solution containing TTX (10^{-6} M), a high K^+-evoked release of Glu was suppressed to 35% of that observed in control medium, with no change in the spontaneous release (Figure 2a). TTX prevents nerve conduction by blocking the sodium channels (Narahashi, 1974), therefore, the insensitive component of Glu release to TTX was presumably due to direct depolarization of the nerve terminals (Blaustein *et al.*, 1972; Alberts *et al.*, 1981; 1982). Subsequently, a series of experiments were carried out to investigate possible effects of a specific dopamine receptor agonist and antagonist on high K^+-evoked Glu release. LY-171555 inhibited the high K^+-evoked Glu release by 44%. (−)-Sulpiride (10^{-7} M) reversed the inhibition of high K^+-evoked Glu release induced by LY-171555 (10^{-6} M) (Figure 2b).

Stimulus-induced increase in intracellular level of Ca^{2+}

When the quin2-loaded slices were stimulated by rectangular pulses (2 mA, 2.5 ms, 5 Hz, train of 25 pulses), the relative fluorescence intensity was increased within 1 s after the onset of stimulation and reached a maximum about 5 s later (Figure 3A, a and d). The electrical stimulation produced no change in fluorescence intensity in control slices incubated without quin2/AM (Figure 3C). To determine whether the

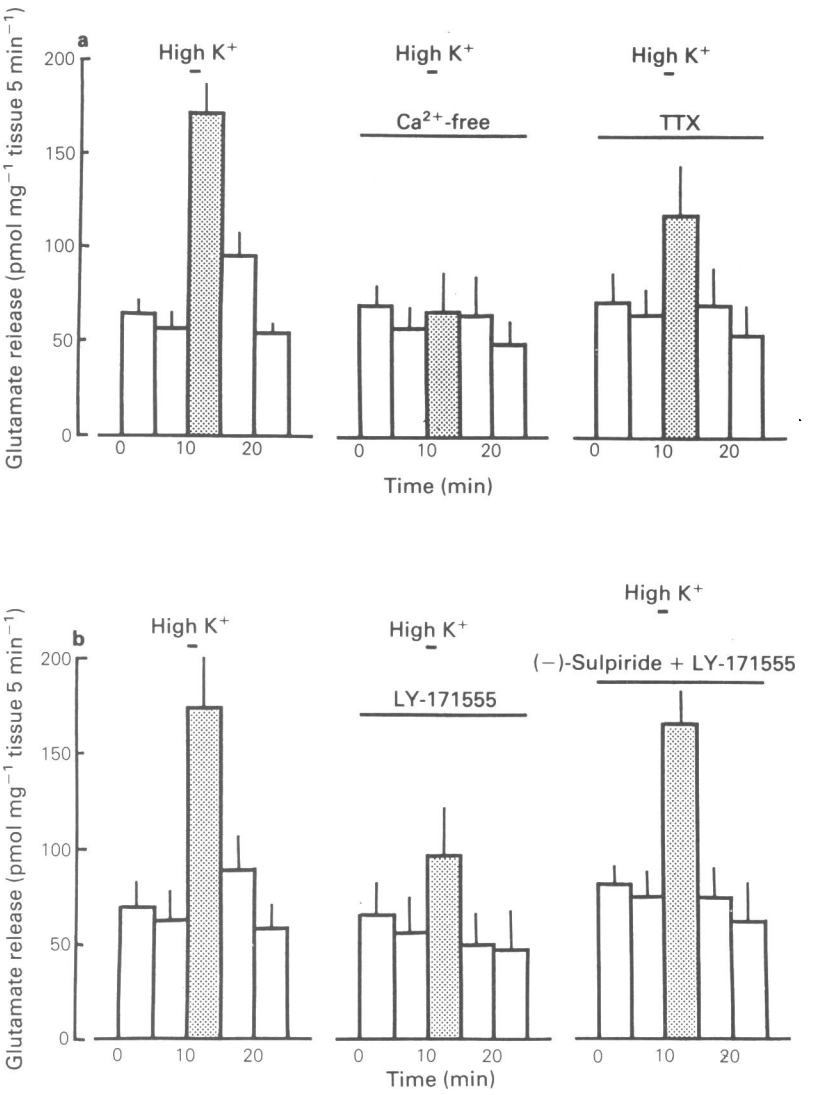

Figure 2 (a) Effect of Ca^{2+}-free medium or tetrodotoxin (TTX, 10^{-6} M) on high K^+-evoked endogenous glutamate release from neostriatal slices. (b) Effect of LY-171555 (10^{-6} M) and (−)-sulpiride (10^{-7} M) on high K^+ evoked endogenous glutamate release. In (a) and (b) each column represents the mean from 4 experiments and vertical lines indicate s.e.mean.

elevation of fluorescence intensity induced by electrical stimulation was dependent on extracellular Ca^{2+}, we investigated the effect of removal of Ca^{2+} from the medium on the elevation of fluorescence in quin2-loaded slices. As shown in Figure 3A and Table 2, the elevation of fluorescence intensity gradually decreased, depending on time after the removal of Ca^{2+} from the medium. After 15 min, the response was attenuated to 7.3% of that observed in the presence of extracellular Ca^{2+} (2 mM). This reduction in response observed in Ca^{2+}-free KRB solution rapidly diminished after re-exposure to normal KRB solution (Figure 3A, d). Perfusion of KRB solution containing TTX (10^{-6} M) for 3 min abolished the increase of fluorescence intensity (Figure 3B and Table 2).

When the quin2-loaded slices were perfused with high K^+ (30 mM) medium, the fluorescence intensity increased within 10 s and reached a maximum about

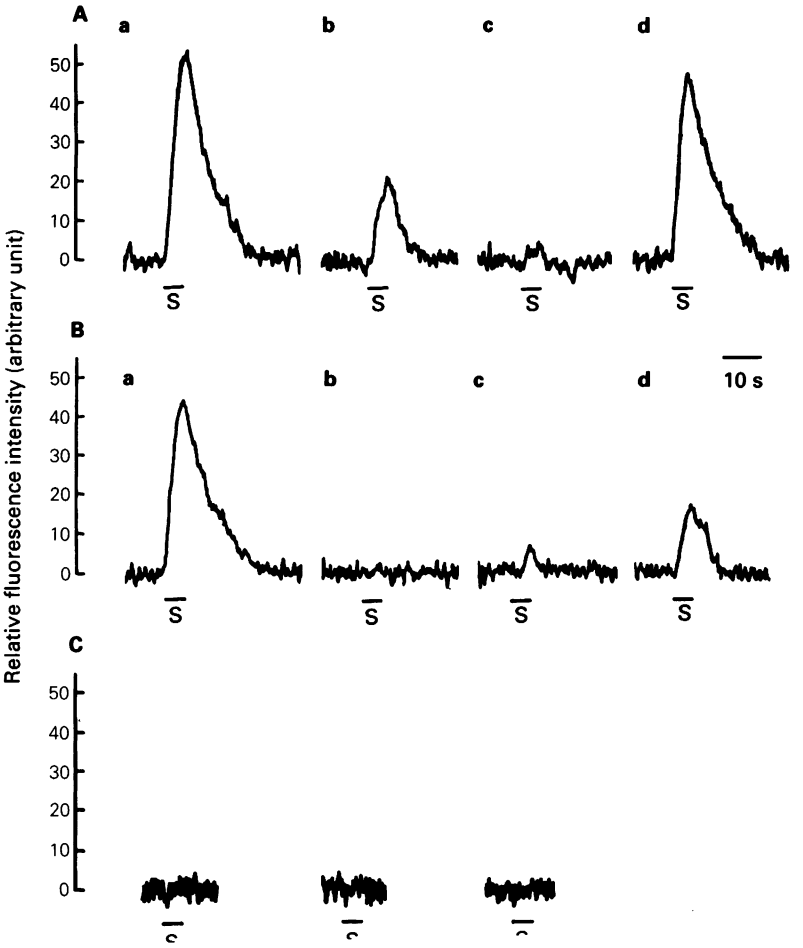

Figure 3 The continuous monitoring of relative changes of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in neostriatal slices following electrical stimulation, using the Ca^{2+}-selective fluorescent indicator, quin2. Electrical stimulation (S, 2 mA, 5 Hz, 2.5 ms) of slices was applied as a train of 25 pulses for 5 s. (A) Effect of extracellular Ca^{2+} concentration on electrically stimulated elevation of fluorescence intensity as a measure of $[Ca^{2+}]_i$. (a) Normal Krebs-Ringer bicarbonate (KRB) solution. (b and c) Suppression of response at 5 and 15 min, respectively, after application of Ca^{2+}-free medium containing EGTA (10^{-4} M). (d) Recovery of response suppressed by Ca^{2+}-free medium at 5 min after re-exposure to control medium. (B), (a) Elevation of fluorescence as a measure of the increase of $[Ca^{2+}]_i$ induced by electrical stimulation in control medium. (b) Effect of tetrodotoxin (TTX, 10^{-6} M) on stimulated elevation of fluorescence intensity. Fluorescence intensity was recorded at 3 min after superfusing the slices with KRB solution containing 10^{-6} M TTX. (c and d) Recovery of response blocked by TTX at 5 and 15 min after replacing the medium containing TTX with control medium. (C) Fluorescence intensity in neostriatal slices incubated without quin 2/AM. Each recording shown is representative of data from 4 experiments.

60 s later (Figure 4). The high K^+-evoked elevation of fluorescence intensity was reduced to 8.8% of the control 15 min after removal of Ca^{2+} from the medium (Table 2). TTX (10^{-6} M) reduced the high K^+ (30 mM)-induced increase of fluorescence intensity to 68.7% of the control (Figure 4 and Table 2).

Effect of dopamine receptor agonists and an antagonist on $[Ca^{2+}]_i$

LY-171555 (10^{-8}–10^{-5} M) inhibited, in a concentration-dependent manner, the increase of $[Ca^{2+}]_i$ induced by electrical stimulation of the neostriatal

Table 2 Effect of Ca^{2+}-free medium or tetrodotoxin (TTX) on the increase of $[Ca^{2+}]_i$ induced by depolarizing stimuli

Treatment	*Relative fluorescence intensity* (arbitary units)	*% of control*
(A) Electrical stimulation-induced increase of $[Ca^{2}]_i$		
None	48.9 ± 2.2 $(n = 10)$	
Ca^{2+}-free medium‡		
5 min	20.9 ± 1.9* $(n = 4)$	42.0
15 min	3.55 ± 0.5* $(n = 4)$	7.3
TTX (10^{-6} M)	2.15 ± 0.3* $(n = 4)$	4.4
(B) High K^+-induced increase of $[Ca^{2+}]_i$		
None	35.1 ± 3.1 $(n = 5)$	
Ca^{2+}-free medium‡		
15 min	3.10 ± 2.3* $(n = 5)$	8.8
TTX (10^{-6} M)	24.1 ± 2.7 $(n = 5)$	68.7

Electrical stimulation was at 2 mA, 5 Hz, 2.5 ms for 5 s. Slices were also stimulated by application of high K^+ (30 mM) medium for 30 s. ‡Slices were superfused with Ca^{2+}-free KRB solution containing 10^{-4} M EGTA. *Significantly different from each control ($P < 0.05$).

slices (Figure 5). As is evident in the response curve, the concentration required to induce the half-maximal response (EC_{50}) was 4×10^{-7} M. The maximal inhibitory effect of LY-171555 at 3×10^{-5} M was a $35 \pm 3\%$ ($n = 7$) reduction in the control values. Apomorphine also inhibited, in a concentration-dependent manner, the increase of $[Ca^{2+}]_i$ induced by electrical stimulation and the EC_{50} was 2.2×10^{-7} M (Figure 5b).

In contrast, the specific D_1-dopamine receptor agonist, SK&F 38393 did not affect the increase of $[Ca^{2+}]_i$ induced by electrical stimulation (Figure 5b). When (−)-sulpiride (10^{-7} M) was perfused 15 min before and during the application of LY-171555 (10^{-6} M), the inhibitory effect of the latter on the electrically stimulated increase of $[Ca^{2+}]_i$ was reversed by $91 \pm 3\%$ of the control level. In the presence of (−)-sulpiride (10^{-7} M), the inhibitory effect of apomorphine (10^{-6} M) was also reversed by $96 \pm 1.6\%$. (−)-Sulpiride alone slightly reduced the increase of $[Ca^{2+}]_i$ induced by electrical stimulation, but not significantly.

LY-171555 (10^{-6} M) significantly inhibited the increase of $[Ca^{2+}]_i$ induced by high K^+ medium. This inhibitory effect of LY-171555 on the high K^+-induced increase of $[Ca^{2+}]_i$ was antagonized by (−)-sulpiride (10^{-7} M) (Figure 6 and Table 1).

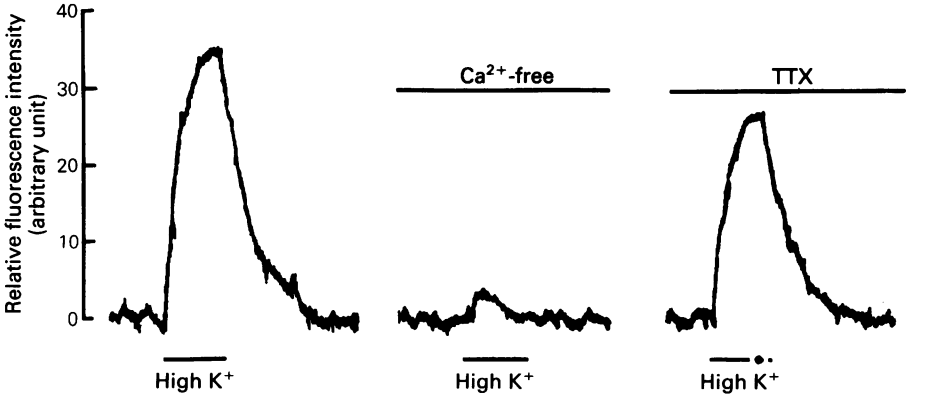

Figure 4 Effect of Ca^{2+}-free medium or tetrodotoxin (TTX, 10^{-6} M) on the increase of $[Ca^{2+}]_i$ induced by application of high K^+ (30 mM) medium to neostriatal slices for 30 s. Representative data of 5 experiments are illustrated.

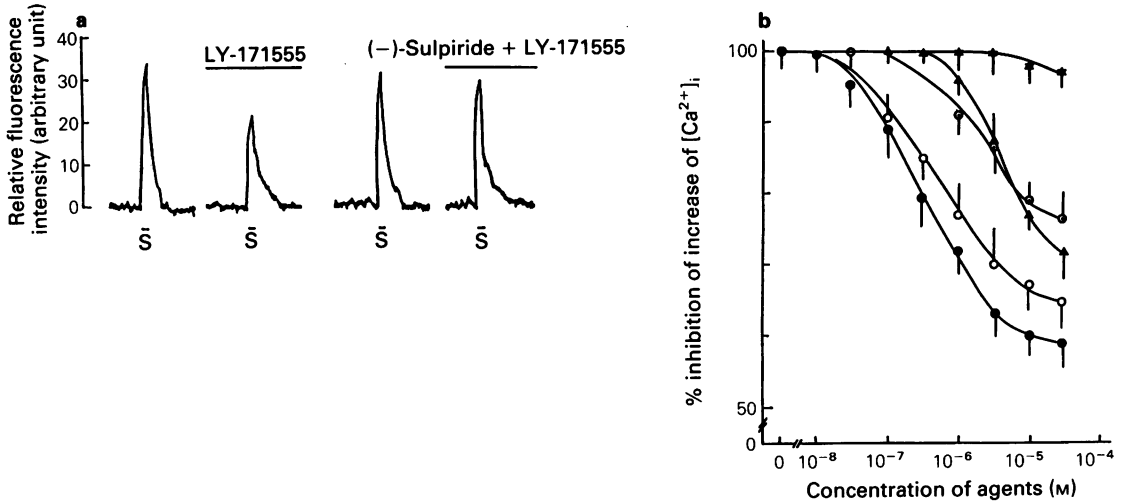

Figure 5 (a) Effect of LY-171555 (10^{-6} M) and (−)-sulpiride (10^{-7} M) on electrically stimulated increase of $[Ca^{2+}]_i$ in neostriatal slices. LY-171555 (10^{-6} M) was added to the medium 15 min before stimulation. (−)-Sulpiride was added 5 min before application of LY-171555 (10^{-6} M) and then stimulated. S: indicates electrical stimulation (2 mA, 5 Hz, pulse duration 2.5 ms for 5 s). Representative data of 4 experiments are shown. (b) Concentration-response curve for the effect of dopamine receptor agonists and an antagonist on electrically stimulated increase of $[Ca^{2+}]_i$: (○) LY-171555, (●) apomorphine, (⊙) (−)-sulpiride (10^{-7} M) plus LY-171555, (▲) (−)-sulpiride (10^{-7} M) plus apomorphine and (★) SK&F 38393. Each point is mean % stimulated increase of $[Ca^{2+}]_i$ (compared to control medium) from 4–7 experiments and vertical lines indicate s.e.mean.

As shown in Figure 7, at 5 min following the omission of extracellular Ca^{2+}, the electrical stimulation-induced increase of $[Ca^{2+}]_i$ was reduced to 46.1% of that in the presence of Ca^{2+}. LY-171555 (10^{-6} M) reduced this evoked increase of $[Ca^{2+}]_i$ in the absence of Ca^{2+} to 18% of that in the presence of Ca^{2+} (Table 1).

Discussion

The two main objectives of the present work were to obtain evidence for: (1) D_2-dopamine receptor-mediated inhibition of depolarizing stimuli-induced elevation of $[Ca^{2+}]_i$ and (2) presynaptic D_2-dopamine receptor-mediated inhibitory control of ACh release through its inhibitory action on the elevation of $[Ca^{2+}]_i$.

The release of [^{3}H]-ACh from the striatal slices induced by depolarizing stimuli was shown to be dependent on extracellular Ca^{2+} and sensitive to TTX, thereby indicating that the released [^{3}H]-ACh was of neuronal origin. The release of [^{3}H]-ACh by depolarizing stimuli was inhibited by an agonist of the D_2-receptor, LY-171555, and this effect was antagonized by (−)-sulpiride. These results indicate the existence of a presynaptic D_2-dopamine receptor which mediates inhibitory control of ACh release from neostriatal slices, as described by Stoof & Kebabian (1982), Cubeddu & Hoffmann (1983) and Baud *et al.* (1985). Stoof & Kebabian (1982) showed that, in rat striatal slices, the stimulation of the D_2-dopamine receptor reduces cyclic AMP formation brought about by stimulation of the D_1-dopamine receptor. The D_2-dopamine receptor-mediated inhibition of cyclic AMP formation occurred in both the presence and absence of extracellular Ca^{2+}. However, drugs stimulating cyclic AMP formation did not affect the D_2-dopamine receptor-mediated inhibition of [^{3}H]-ACh release. From these observations they concluded that the D_2-dopamine receptor-mediated inhibition of cyclic AMP formation does not participate in neurotransmitter release. There are several possible explanations for the receptor-mediated presynaptic inhibition of neurotransmitter release: (1) the inhibition of Ca^{2+} influx, (2) the inhibition of Ca^{2+} release from the store or (3) the decreased rate of synthesis of neurotransmitters. The evoked neurotransmitter release is likely to occur mainly by exocytosis, an event triggered by an increase in the free cytoplasmic Ca^{2+} concentration within the varicosities of the axon terminals (Miledi & Parker, 1981). We found that the Ca^{2+} selective indicator quin 2 can be used to monitor $[Ca^{2+}]_i$ in brain slices, as was noted in the case of synaptosomes (Ashley *et al.*, 1984; Meldolesi *et al.*, 1984) and other free cells (Tsien *et al.*, 1982a,b; Schofield, 1983;

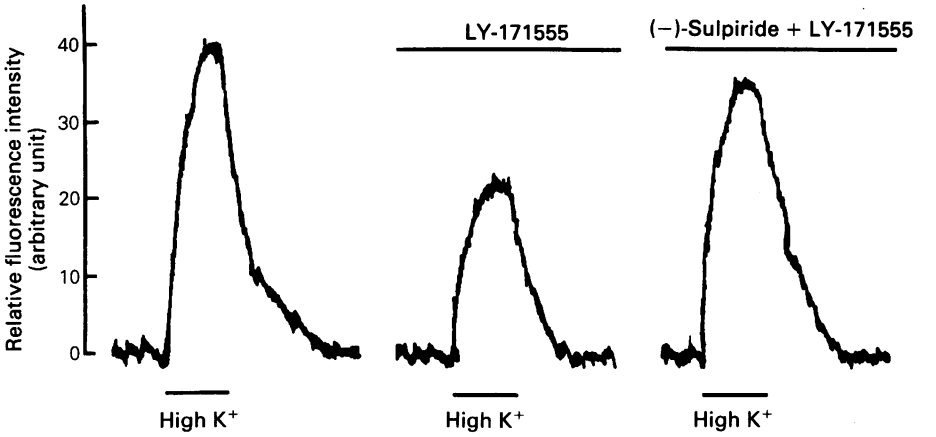

Figure 6 Effect of LY-171555 (10^{-6} M) and (−)-sulpiride on high K^+ evoked increase of $[Ca^{2+}]_i$. Stimulation was performed by superfusing slices for 30 s with KRB solution containing 30 mM K^+, which was prepared by an equimolar replacement of NaCl by KCl. Representative data of 4 determinations are shown. LY-171555 (10^{-6} M) was added to the medium 15 min before high K^+ medium. (−)-Sulpiride (10^{-7} M) was applied 5 min before the application of LY-171555 (10^{-6} M).

Hallam *et al.*, 1984; Rorsman *et al.*, 1984; Virgilio *et al.*, 1984). Depolarizing stimulation of neostriatal slices loaded with quin 2 produced an elevation in fluorescence intensity and this was suppressed when Ca^{2+} was removed from the medium. We also observed that the elevation in fluorescence intensity was abolished by treatment with 5 mM $CoCl_2$-containing medium (Saijoh *et al.*, 1985). Since Co^{2+} at the concentration used will block the Ca^{2+} channel, these results indicate that the stimulated increase in fluorescence intensity in quin 2 loaded slices can be attributed to an increase of $[Ca^{2+}]_i$. TTX blocked the increase in fluorescence intensity induced by electrical stimulation and this compound inhibits neuronal conduction by blocking Na^+ channels (Narahashi, 1974). Therefore, our findings suggest that the depolarization stimulated increase in fluorescence intensity is of neuronal origin. The depolarizing stimuli-induced increase of $[Ca^{2+}]_i$ can probably be explained by changes associated with both Ca^{2+} influx from extracellular fluid and Ca^{2+} release from intracellular store sites.

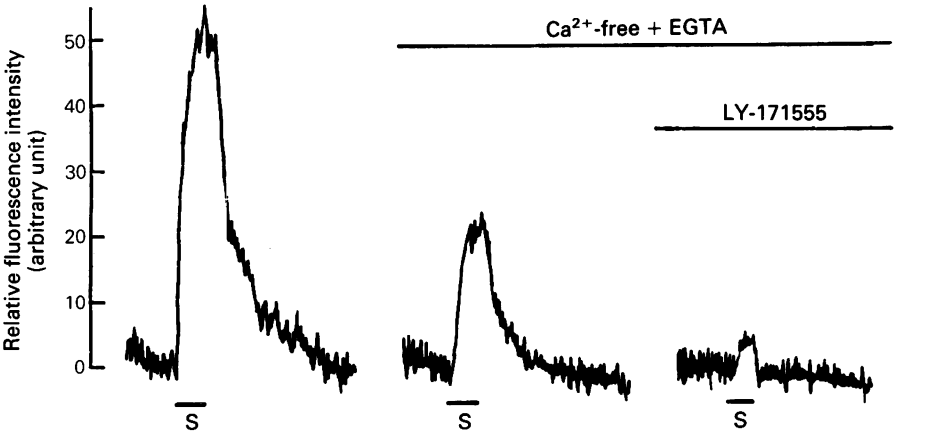

Figure 7 Effect of LY-171555 on the electrically stimulated increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. Ca^{2+}-free medium containing EGTA (10^{-4} M) was perfused 5 min before electrical stimulation (s). LY-171555 (10^{-6} M) was applied to the medium 5 min before electrical stimulation. Electrical stimulation (2 mA, 5 Hz, 2.5 ms) was applied as a train of 25 pulses for 5 s. Representative data of 5 experiments are illustrated.

In the presence of Ca^{2+} in the media, LY-171555 suppressed the rise of $[Ca^{2+}]_i$ induced by electrical stimulation, or by high K^+ medium, an event antagonized by (−)-sulpiride. Apomorphine also reduced the stimulation-induced increase of $[Ca^{2+}]_i$, and this inhibitory effect was reversed by (−)-sulpiride. In contrast, a specific D_1-receptor agonist, SK&F 38393 did not affect the stimulus-induced increase in $[Ca^{2+}]_i$. The EC_{50} of LY-171555 for its inhibitory effect on the evoked [^{3}H]-ACh release was about 2×10^{-7} M and this value was similar to the EC_{50} of LY-171555 for its inhibitory effect on the increase in $[Ca^{2+}]_i$ induced by electrical stimulation. A high correlation ($r = 0.997$, statistically significant, $P < 0.05$) was observed between values of % inhibition of [^{3}H]-ACh release and those of % inhibition of $[Ca^{2+}]_i$ elevation in the presence of LY-171555, at concentrations of 3×10^{-8} M to 3×10^{-6} M. These data show that activation of the D_2-dopamine receptors reduces the release of neurotransmitter by inhibiting the rise of $[Ca^{2+}]_i$.

There are at least two mechanisms by which the D_2-receptor agonist could inhibit the elevation of $[Ca^{2+}]_i$, by suppressing Ca^{2+} influx or Ca^{2+} release from the intracellular store. The $[Ca^{2+}]_i$ and ACh release did not respond to depolarizing stimuli 15 min after superfusing the slices with Ca^{2+}-free KRB solution containing EGTA (10^{-4} M), thereby suggesting that, under this condition, there is depletion of Ca^{2+} from the intracellular store sites. The electrically stimulated elevation of $[Ca^{2+}]_i$ was 46.1% of the control at 5 min after superfusing the slices with Ca^{2+}-free KRB solution containing EGTA (10^{-4} M). Therefore, it is thought that elevation of $[Ca^{2+}]_i$ is due to mobilization of the ion from the intracellular store sites, under these circumstances. LY-171555 (10^{-6} M) inhibited the depolarization-induced elevation of $[Ca^{2+}]_i$ by 23% of the control in the presence of extracellular Ca^{2+} and reduced the electrically stimulated elevation of $[Ca^{2+}]_i$ at 5 min after superfusing with Ca^{2+}-free KRB solution containing EGTA (10^{-4} M) to 18% of the control. In other words LY-171555 inhibited the $[Ca^{2+}]_i$ elevation by 28.1% (that is 46.1% minus 18.0%) of the control. These results suggest that LY-171555 suppresses intracellular Ca^{2+} mobilization from internal store sites, rather than the transmembrane Ca^{2+} influx. As shown in Figures 1 and 5, EC_{50} values of LY-171555 for the inhibition of both the electrically stimulated elevation of $[Ca^{2+}]_i$ and [^{3}H]-ACh release were of the order 10^{-7} M which is near the IC_{50} of its inhibition of [^{3}H]-spiperone binding to the brain membrane found by Lehmann *et al.* (1983). The inhibitory effect of LY-171555 was antagonized by (−)-sulpiride. These findings suggest that LY-171555 probably inhibits the stimulated elevation of $[Ca^{2+}]_i$ through D_2-dopamine receptor activation. This is supported by electrophysiologically-obtained data that the Ca^{2+}-dependent action potential, in a prolactin secreting cell line, was blocked by the D_2-dopamine receptor agonist, RU 24213, and that this inhibitory effect was antagonized by haloperidol (Dufy *et al.*, 1979). It has also been shown that dopamine inhibits the increase in $[Ca^{2+}]_i$, due to TRH-induced release of Ca^{2+} from intracellular store sites, in bovine anterior pituitary cells (Schofield, 1983). It is widely accepted that prolactin release from the anterior pituitary gland is under tonic inhibitory dopaminergic control. The stimulation of D_2-dopamine receptors is thought to inhibit prolactin release by inhibiting the Ca^{2+}-dependent process. In the case of prolactin release from the pituitary gland, the role of intracellular cyclic AMP has not been elucidated.

Canonico *et al.* (1982) showed that stimulation of the D_2-dopamine receptor inhibits ^{32}P incorporation into phosphatidylinositol in the rat anterior pituitary gland. They suggested that a decrease in phosphatidylinositol cleavage and turnover may be involved in the inhibitory regulation of prolactin release by dopamine. The hydrolysis of phosphatidylinositol is thought to be an early event associated with the activation of the receptor and to be induced by depolarization. Neuronal depolarization-induced phosphatidylinositol turnover leads to production of diacylglycerol and inositol trisphosphate. The former activates protein kinase C and the latter mobilizes intracellular Ca^{2+} from internal store sites (Berridge & Irvine, 1984; Nishizuka, 1984). Hence the D_2-dopamine receptor-mediated inhibitory effect on intracellular Ca^{2+} mobilization in our study may be due to the inhibition of phosphatidylinositol turnover induced by electrical stimulation. The molecular mechanisms involved are the subject of ongoing studies.

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