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DECREASED CALCIUM UPTAKE BY RAT FUNDAL STRIPS AFTER PRETREATMENT WITH NEURAMINIDASE OR LSD *IN VITRO*

EFFECT OF SEROTONIN, D-AMPHETAMINE AND ELEDOISIN ON THIS UPTAKE*

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Abstract—Treatment with neuraminidase plus EDTA diminished ^{45}Ca uptake by fundal strips; this decrease occurred also in the presence of 5-hydroxytryptamine (5-HT), D-amphetamine (DA) and eledoisin. At the same time, 5-HT-, DA- and eledoisin-induced contractions were blocked to varying extents. Lysergic acid diethylamide (LSD) also caused a decrease in ^{45}Ca uptake; the decrease remained sustained also when applying 5-HT or DA, and contractions due to these drugs were completely blocked. In opposite, eledoisin addition increased ^{45}Ca uptake after LSD, and also it was able to provoke muscle contractions.

From these results, drug-induced contractions are envisaged as occurring in 4 stages: (1) drug–receptor interaction; then (2) calcium–ganglioside interaction; then (3) calcium transport leading to (4) contraction. It is concluded that gangliosides may be a functional part of carrier molecules rather than portions of drug receptors. Moreover, the results obtained with neuraminidase and LSD lead to the hypothesis that they act possibly by interfering with the active transfer of calcium-ions to the contractile structures: the former by destroying the calcium transport sites and the latter by blocking the receptor sites where agonists act.

THE PRESENCE of proteins in the structure of cholinergic,[1–7] adrenergic,[8–10] and serotoninergic receptors[4,5,11–14] is now widely accepted.

Woolley,[15] and Woolley and Gommi[16,17] early emphasized the importance of gangliosides in the structure of the 5-HT receptor. Several recent reports have also demonstrated a striking relationship between drug-induced smooth muscle contraction and the integrity of the tissue gangliosides.[18–23] We showed in a previous paper[23] that gangliosides were not involved in the binding of ^{14}C-5-HT to 5-HT receptors and ^{3}H-DA to DA-receptors. That is, enzyme breakdown of gangliosides by neuraminidase was able to prevent drug-induced contraction, but was unable to affect the tissue uptake of both labelled drugs.

Woolley and coworkers suggested that 5-HT, when bound to the receptor, stimulated the transport of extracellular calcium through the cell membrane, in order to make contraction possible.[24–26] Hurwitz and Joiner[27] and others have also suggested carrier or saturable transport systems for the transfer of extracellular Ca-ions into the cell.

The present work was intended to determine whether it is possible to dissociate

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drug–receptor interaction from calcium transport and muscle contraction. ^{45}Calcium uptake by rat fundal strips was measured in normal tissue before and after the addition of 5-HT, DA and eledoisin. At the same time muscle contractions were recorded. The same parameters were measured after treating the tissue with LSD or neuraminidase.

MATERIALS AND METHODS

Tissue preparation

Rat fundal strips, mucosa removed, were prepared according to Offermeier's modification[28] of Vane's method.[29] The tissues were weighed wet, mounted in a 10 ml organ bath and bathed in a modified Ringer solution at 37° gassed with pure O_2. The composition of the modified Ringer solution was (g/l.): NaCl 9·0—KCl 0·42—$NaHCO_3$ 0·5—glucose 0·5—$CaCl_2$ 0·06. ^{45}Calcium-containing Ringer solution consisted of the same salts but in addition contained 5·0 mc of ^{45}Ca/l. The extra calcium added was approximately 8×10^{-4} g/l. which *per se* was not able to cause contraction of the fundal strip. Before testing, the fundal strips were allowed to relax for 30–60 min, with bathing fluid changes every 15 min. After this period the tissues were stable and showed constant responses to drugs. Control tissues, or tissues treated with neuraminidase or LSD (see below) were then exposed to ^{45}Ca-Ringer with or without agonists, so that simultaneous measurements of Ca uptake and contraction could be made.

Radioisotope experiments

In these experiments the method of Bianchi and Shanes[30] was used. The Ringer solution was washed out and replaced by Ringer solution containing ^{45}Ca. After 2 and 30 min the strips were removed from the radioactive bathing medium, rinsed by rapidly dipping in unlabelled Ringer and blotted on filter paper.

The times of 2 and 30 min were chosen to correspond to the approximate period of contraction (2 min), and a well sustained contraction (30 min) after agonist addition.

Estimation of the radioactive calcium

^{45}Calcium was determined with a Packard Tri-Carb Liquid Scintillation Spectrometer (Modell 2211), by the method of Humphreis.[31] Each tissue sample was oxidized by heating with nitric acid. The calcium was precipitated as the oxalate, and then converted to calcium perchlorate by a two-stage incineration process with perchloric acid. The residue from the second incineration was dissolved in 2 ml of tributylphosphate (TBP) and 4 ml of scintillation solution, made up of 3 g of 2,5-diphenyloxazole (PPO) in 1000 ml of toluene. The mixture was transferred to counting vials, containing 6 ml of scintillator solution. Samples containing tissue with and without known amounts of radioactivity (standards) were run in the same way to determine the background and counting efficiency (established as 52–58 per cent). The counts per min per sample (counts/min sample) for each tissue sample were corrected for efficiency and expressed as disintegrations per min per mg of wet organ weight (dis./min/mg).

Ganglioside hydrolysis by neuraminidase

The method used was that of Woolley and Gommi.[17] Purified neuraminidase derived from *Clostridium perfringens* (0·05 micromolar Units per mg), was applied

twice to the tissue as follows: 0·5 ml of neuraminidase solution (1 mg in 5 ml of modified Ringer solution at 4°) was mixed with 4·5 ml of a solution of ethylendiaminetetracetate disodium salt (EDTA), pH 7·3; 0·5 ml of the mixture (neuraminidase -EDTA) was immediately added to the 10 ml tissue bath. The enzyme–EDTA mixture was allowed to act for 10 min, and was then washed out. Five min after washing, calcium chloride (2 mg) was added for 90 sec and then the tissue was washed for 60 min; 0·5 ml of neuraminidase plus EDTA (N-EDTA) freshly mixed) was added a second time for 10 min. The tissue was washed for 5 min, $CaCl_2$ (2 mg) added for 90 sec, and then washed for 10 min again. The tissue was ready for the assay.

Preincubation with LSD

Fundal strips were preincubated with lysergic acid diethylamide (LSD), 0·5 μg/ml for 1 hr, a dose and time which irreversibly blocked 5-HT and DA-induced contractions. After the incubation period, the strips were washed with fresh Ringer.

Chemicals

D-amphetamine sulphate and 5-hydroxytryptamine creatinine sulphate were purchased from Merck-Germany. Eledoisin was kindly supplied by Dr B. Camerino-Farmitalia, Milano. Lysergic acid diethylamide tartrate (Delysid) was purchased from Sandoz, Switzerland. Drug concentrations are expressed as the free base. Neuraminidase type V (*Clostridium perfringens*) was purchased from Sigma, U.S.A. ^{45}Calcium chloride (6 c/g Ca^{2+}) was purchased from the Radiochemical Centre, Amersham, England.

RESULTS

^{45}Calcium was shown to be taken up by both quiescent and contracting smooth muscle. In control strips (no drug added), the accumulation of calcium by the tissue

TABLE 1. EFFECT OF 5-HT, DEXAMPHETAMINE AND ELEDOISIN ON ^{45}CALCIUM UPTAKE BY NORMAL RAT FUNDAL STRIPS, AT 2 AND 30 min AFTER INCUBATION WITH ^{45}CALCIUM*

Drugs	$^{45}CaCl_2$*	Incubation time (min)	Normal strips (dis./min/mg ± S.E.)	No. of strips	Significance of differences† vs. control strips	State of contraction
None	+	2	8309·0 (± 420·9)	6	—	absent
		30	15,240·5 (±1491·5)	6	—	absent
5-HT	+	2	6792·3 (± 192·7)	6	P<0·01 (D)	maximal
10^{-4} M		30	14,827·8 (±1204·8)	6	n.s.	sustained
D-amphetamine	+	2	7270·7 (± 719·3)	6	n.s.	maximal
10^{-4} M		30	14,068·0 (± 983·5)	6	n.s.	sustained
Eledoisin	+	2	7781·2 (± 542·8)	6	n.s.	maximal
0·05 μg/ml		30	16,488·7 (±1685·7)	6	n.s.	sustained

* ^{45}Ca: 5 μc/0·8 μg/ml.
† Student's *t*-test.
(D) Decrease in ^{45}Ca compared with control strips.

was observed to progress from 2 until 30 min (Table 1). At 2 min, both dexamphetamine and eledoisin elicited a slight but non-significant decrease in the ^{45}calcium content of the contracted fundal strips, compared with control tissues.

By contrast, 5-HT significantly decreased (18·3 per cent) the amount of radioactivity present in the contracted strips. All three agonists caused maximal contractions at 2 min, and sustained contractions at 30 min.

At 30 min, none of the drugs tested provoked significant changes in tissue radioactivity, in comparison with the controls.

The effect of the breakdown of gangliosides by N-EDTA on drug-induced variations of radioactivity is shown in Table 2.

At 2 min, the ^{45}Ca content of the ganglioside-destroyed tissues was unaffected by the addition of 5-HT, DA and eledoisin (compared to controls), although the contractions induced by these agonists were inhibited to varying extents as shown in a previous paper.[23]

At 30 min, while 5-HT and DA did not induce significant variations of the ^{45}Ca uptake, eledoisin decreased the radioactivity by 23·7 per cent, in comparison with control tissues.

When comparing the N-EDTA treated strips with normal (untreated) strips, it was seen that at 2 min, the ^{45}Ca present in the tissue was markedly decreased by enzyme treatment (37·4 per cent). At 30 min, when comparing N-EDTA treated tissue with normal strips, it was observed that there was no significant effect on the radioactivity of control, DA and eledoisin incubated strips, although there was a 22·6 per cent increase due to 5-HT

The addition of 5-HT to LSD-preincubated tissue decreased calcium uptake at 2 min compared to the control strips (Table 3). DA elicited a smaller and not significant decrease, while eledoisin caused an increase in calcium uptake. The 5-HT- and DA-provoked contractions were completely abolished while the eledoisin-induced contraction was unaffected by LSD. Moreover, at 30 min, addition of 5-HT was without effect while DA and eledoisin had an increasing effect, in comparison with controls.

At 2 min, a 34·2 per cent decrease in calcium uptake by control strips of LSD-treated tissue compared with control strips of normal tissue was found. Addition of 5-HT or DA significantly decreased the ^{45}Ca content, in comparison with the normal fundus at 2 min (15 and 10 per cent decrease respectively). By contrast, no significant change was seen after eledoisin.

At 30 min, after LSD preincubation, no significant effect was observed on the tissue radioactivity as compared to normal tissue. At 30 min, the agonists tended generally to increase calcium uptake in LSD treated tissue as compared with normal tissue. The increase was significant only for DA, however.

When comparing LSD-treated strips with N-EDTA treated strips it was found that there was no statistically significant difference in calcium uptake by controls of these two tissues.

At 2 min calcium uptake was further decreased after 5-HT or DA in LSD strips, while eledoisin stimulated an increase in calcium uptake by LSD strips.

At 30 min both DA and eledoisin stimulated calcium uptake while 5-HT had no significant effect on calcium uptake by LSD strips compared with N-EDTA strips.

TABLE 2. EFFECT OF 5-HT, DEXAMPHETAMINE AND ELEDOISIN ON ^{45}CALCIUM* UPTAKE BY NEURAMINIDASE PLUS EDTA TREATED RAT FUNDAL STRIPS

Drugs	$^{45}CaCl_2$*	Incubation time (min)	Neuraminidase-treated strips (dis./min/mg ± S.E.)	No. of strips	Significance of differences† vs. normal strips	Significance of differences† vs. control strips‡	State of contraction§
None	+	2	5201·8 (±538·0)	5	P < 0·005 (D)	—	none
		30	17,822·0 (±669·6)	6	n.s.	—	none
5-HT	+	2	5510·0 (±196·7)	5	P < 0·005 (D)	n.s.	inhibited
10^{-4} M	+	30	18,173·0 (±813·0)	6	P < 0·05 (I)	n.s.	slow contracture
D-amphetamine		2	6437·8 (±162·9)	5	n.s.	n.s.	partial inhibit.
10^{-4} M		30	16,504·0 (±880·9)	6	n.s.	n.s.	slow contracture
Eledoisin	+	2	5432·0 (±334·6)	6	P < 0·005 (D)	n.s.	partial inhibit.
0·05 μg/ml		30	13,601·2 (±279·1)	6	n.s.	P < 0·001 (D)	slow contracture

* ^{45}Ca: 5 μc/0·8 μg/ml.
† Student's *t*-test.
‡ Neuraminidase-treated, $^{45}CaCl_2$-added strips.
§ See also Ref. 23.
(D) Decrease in ^{45}Ca in N-EDTA treated strips.
(I) Increase in ^{45}Ca in N-EDTA treated strips.

TABLE 3. EFFECT OF 5-HT, DEXAMPHETAMINE AND ELEDOISIN ON ^{45}CALCIUM* UPTAKE BY RAT FUNDAL STRIPS AFTER INCUBATION WITH LSD†

Drugs	$^{45}CaCl_2$*	Incubation time (min)	LSD treated strips (dis./min/mg ± S.E.)	No. of strips	Significance of differences‡ vs. normal strips	vs. control§ strips	vs. N-EDTA treated strips	State of contraction
None	+	2	5468·2 (±373·7)	6	P < 0·001 (D)	—	n.s.	absent
		30	17,068·0 (±335·6)	6	n.s.	—	n.s.	absent
5-HT	+	2	4369·2 (±230·7)	5	P < 0·001 (D)	P < 0·05 (D)	P < 0·01 (D)	blocked
10^{-4} M		30	17,097·4 (±591·7)	5	n.s.	n.s.	n.s.	blocked
D-amphetamine	+	2	4541·8 (±152·6)	5	P < 0·01 (D)	n.s.	P < 0·001 (D)	blocked
10^{-4} M		30	19,668·6 (±864·5)	5	P < 0·005 (I)	P < 0·02 (I)	P < 0·05 (I)	blocked
Eledoisin	+	2	7493·7 (±718·7)	6	n.s.	R < 0·05 (I)	P < 0·05 (I)	maximal
0·05 μg/ml		30	18,821·0 (±550·1)	5	n.s.	P < 0·02 (I)	P < 0·001 (I)	sustained

* ^{45}Ca: 5 μc/0·8 μg/ml.
† LSD: 1 hr of incubation with LSD 0·5 μg/ml.
‡ Student's *t*-test.
§ LSD-treated, $^{45}CaCl_2$-added strips.
(D) Decrease in ^{45}Ca in LSD treated strips.
(I) Increase in ^{45}Ca in LSD treated strips.

DISCUSSION

The decrease in calcium uptake by fundal strips after 5-HT although the tissue contracted maximally (Table 1) does not agree with the early findings of Woolley and coworkers[25,26] that 5-HT provoked an increase in calcium uptake by 5-HT-contracted uterine smooth muscle and by the lipid phase of an artificial oil–water system. The decrease occurring under our experimental conditions could be explained by the possibility that ionic translocation during smooth muscle contraction is very fast and bidirectional. At 2 min, a loss of labelled calcium from the cytoplasm towards the extracellular space and bathing medium could occur after the calcium had triggered the contraction.

A similar, although non-significant decrease in radioactivity was noted at 2 min after a contraction induced by DA. At 3 min, the incubation period chosen by Woolley, enough ^{45}Ca may have been accumulated by passive transport to account for his findings. Calcium accumulation due to passive transport could increase with prolongation of the incubation period.

As a second hypothesis, one might suggest that both 5-HT and, to a smaller extent, DA, compete with calcium for some structures of the calcium transport system.

At 30 min, there was no significant variation of the tissue radiocalcium levels after 5-HT, DA or eledoisin, in comparison with the controls. The incubation period of 30 min is indeed long enough to allow equilibration of ^{45}Ca levels between the extracellular and intracellular spaces by both passive and active processes.

It was shown in a previous report that neuraminidase treatment did not affect the uptake of ^{14}C-5-HT or ^{3}H-DA although contractions were blocked to various extents.[23] Thus, the neuraminidase-provoked decrease of ^{45}Ca uptake by agonists and the concomitant prevention of the 5-HT-, DA-, and eledoisin-induced contractions (Table 2) point to the hypothesis that calcium transport processes are altered by ganglioside destruction.

The reservations of Hudgins[32] may be accepted, that changes in total tissue calcium are a partially unreliable index for the estimation of the status of the small fraction of ionized calcium important for the induction and maintenance of contraction. The relations between cytoplasmatic ionized Ca^{2+} and total tissue Ca are indeed variable and the release of Ca^{2+} in the cytoplasm may also take place from membrane stores and endoplasmic reticulum, without need of surplus-uptake of extracellular calcium. Our finding that contractions were also decreased when ^{45}Ca uptake was decreased attenuates however this technical limitation.

The residual calcium uptake after ganglioside destruction both in controls and after agonists, may be considered to be extracytoplasm-bound calcium and/or calcium accumulated by passive processes. The experimental finding that enzyme-breakdown of tissue gangliosides decreased the ^{45}Ca content of the strips, comes as a logical consequence of the acceptance of gangliosides as being a part of the carrier or the carrier itself. Evidence for a relationship between Ca-ions and gangliosides was recently furnished by Kruger and Mendler,[33] who showed that liposolubility of gangliosides was facilitated by calcium-ions in a two-phase solvent system.

The enzyme breakdown of gangliosides decreased calcium uptake to the same extent in control strips or 5-HT treated strips, in comparison with normal tissues. Moreover, addition of 5-HT, DA and eledoisin to neuraminidase-pretreated tissues did not affect uptake at 2 min, in comparison with control strips. Eledoisin caused a

decreased uptake of calcium at 30 min in N-EDTA treated tissues as compared with normal strips. At 2 min it caused a decreased ^{45}Ca uptake when compared to normal tissue but had no effect when compared to control N-EDTA tissue.

It should be noted at this point that N-EDTA treatment does not alter contractile structures. In fact, other agonists, such as acetylcholine or the cholinergic drug furtrethonium were still able to induce normal contractions of the fundal strips after N-EDTA treatment (unpublished results). Heating the tissue to 47° for 20 min also did not affect the contractions induced by these cholinergic drugs.[4,34] These results cannot yet be explained.

Considering now the effects of LSD-preincubation (Table 3), it appears that the ^{45}Ca uptake was strongly decreased as compared to normal, control strips at 2 min. After 5-HT and DA, calcium uptake was significantly decreased compared to normal tissue and contractions were completely blocked. Moreover addition of 5-HT and DA to LSD strips had almost no effect on the radioactivity as compared to control strips. Eledoisin, on the contrary, was still able to contract the fundal strips and there was no decrease in calcium uptake but rather a net, significant increase (Table 3). This is just the opposite of what occurred after N-EDTA, when contractions were partially inhibited and tissue ^{45}Ca diminished.

There is a distinct parallelism between the inability of LSD to block both eledoisin-induced contraction and calcium uptake, and the contemporaneous blockade of 5-HT and DA-provoked contractions, in addition to the diminished uptake of radio-calcium. This suggests that calcium uptake and contraction are strictly related through the mediation of specific receptors or membrane binding sites.

LSD blockade of the 5-HT (and DA) receptors could result in the interruption of the "signal" initiating calcium transport. On the contrary LSD does not act on the eledoisin receptor so that the whole sequence of drug–receptor interaction, calcium transport and contraction may occur. As support for this hypothesis, one may find also a significant eledoisin-induced rise of tissue radioactivity as against LSD-pretreated control strips, and an unchanged ^{45}Ca uptake as against normal eledoisin-contracted tissues.

The results obtained with neuraminidase and LSD lead to the hypothesis that they act possibly by interfering with the active transfer of calcium to the contractile structures: the former by destroying the calcium transport sites and the latter by blocking the receptor sites where agonists act.

Thus two steps in the drug stimulation–contraction are demonstrated:
—the drug–receptor interaction (at 5-HT and DA receptors and eledoisin receptors);
—the carrier molecule–calcium interaction (gangliosides may be an important component, as further support of Woolley's original hypothesis).

The distinction of two steps agrees with the already[23] suggested functional separation between receptor or binding sites for the agonist, and gangliosides. The experimental results thus seem to give some support for Woolley's hypothesis; gangliosides are however viewed as a functional link in the calcium transport chain, rather than as a part of the receptor. Moreover, Woolley's hypothesis considering neuraminidase as "receptor-destroying enzyme" should be modified. Neuraminidase could indeed prevent contraction because of breakdown of the ganglioside-carrier in the calcium transport chain, rather than as a consequence of receptor inactivation.

It is interesting to note that a similar role for gangliosides as carrier of acetylcholine

across presynaptic membrane was already proposed by Burton and Howard.[35] According to these authors, gangliosides could be the carrier for ACh from synaptic vesicles to the presynaptic membrane, and finally to the synaptic cleft, by means of their lipo- and hydro-solubility. This would be feasible through a depolarization-polarization sequence of the presynaptic membrane, that is, alternate electrostatic attractions and repulsions.

A hypothetical scheme for the whole sequence of events starting from drug addition to contraction is shown (Fig. 1).

The agonist (5-HT, DA or eledoisin) interacts with its respective specific receptors. After drug–receptor interaction, changes in polarization of the receptor sites trigger the "approach" of Ca-ions towards the receptor-area and the ganglioside-carrier.

The trigger-pulse for the uptake of calcium could be drug-induced variations of

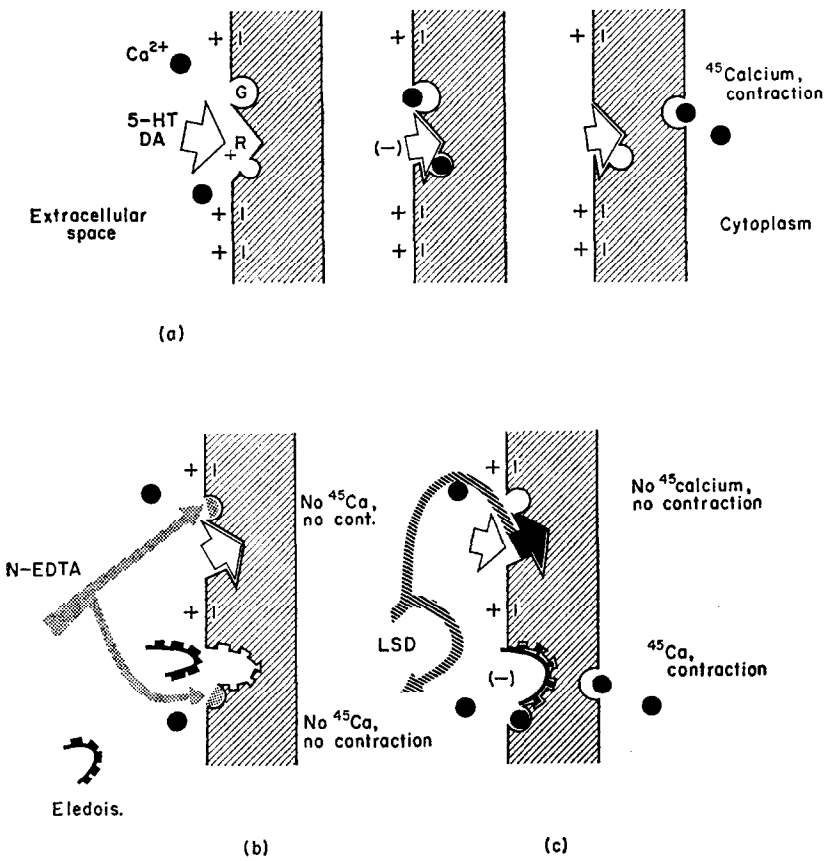

FIG. 1. Hypothetical scheme for the drug addition–contraction sequence. (a) Drug–receptor interaction triggers the "approach" of extracellular ^{45}Ca-ions to carrier-gangliosides (G), by depolarization of the receptor-area (R). The calcium–ganglioside complex reaches the cytoplasm, Ca^{2+} is discharged intracellularly and contraction is then activated. (b) Neuraminidase (N-EDTA) destroys the carrier-ganglioside for Ca-ions and prevents to varying extents 5-HT-, dexamphetamine (DA)-, and eledoisin-induced contractions. The uptake of calcium is decreased. (c) LSD blocks the trigger pulse for the approach of extracellular ^{45}Ca-ions to the 5-HT and DA receptor-area and to gangliosides. On the contrary, LSD affects neither eledoisin-induced contraction nor eledoisin–receptor interaction. The whole sequence as in (a) can thus occur.

the electrostatic equilibrium between the receptor-area and extracellular ions, among them Ca-ions. A considerable amount of calcium ions may be bound to negatively charged extracellular sites such as mucopolysaccharides, proteins, mucoproteins[36] and phospholipids of the cell surface. Changes in membrane electrostatic equilibrium could cause a shift of calcium to ganglioside sites.

The calcium-ganglioside complex then moves across the membrane where Ca^{2+} is discharged.

LSD, by blocking the 5-HT (and DA) receptor sites, prevents the membrane electrostatic changes facilitating Ca-ganglioside interaction. It does not however block eledoisin receptors, and stimulus–contraction coupling proceeds normally.

Neuraminidase destroys the ganglioside carrier while leaving the drug receptor sites essentially intact. Thus drug–receptor interaction occurs, but calcium transport is impaired if not completely blocked.

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