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TRAPPING OF LABELLED LIGANDS IN INTACT CELLS: A PITFALL IN BINDING STUDIES

JEAN-MARIE MALOTEAUX,*‡ ANNIE GOSSUIN,* CHRISTINE WATERKEYN* and PIERRE-M. LADURON†

* International Institute for Cellular and Molecular Pathology, Laboratoire de Chimie Physiologique, Université de Louvain, B 1200 Brussels, Belgium and
† Department of Biochemical Pharmacology, Janssen Pharmaceutica, B 2340 Beerse, Belgium

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Abstract—Binding on/in whole cells seems to be a more appropriate approach for studying receptor sites in physiological conditions. However, certain difficulties encountered throughout the characterization of [^{3}H]spiperone binding in human lymphocytes led us to reconsider this problem. The IC_{50} values of [^{3}H]spiperone binding to human lymphocytes did not correlate with those found in rat striatum; domperidone was inactive in lymphocytes whereas it is one of the most potent dopamine antagonists in rat striatal preparations *in vitro*. In contrast, chloroquine, a lysosomotropic drug, displaced [^{3}H]spiperone at low concentration in intact lymphocytes but did not in the striatum. [^{3}H]Spiperone binding was not displaceable in the membrane preparation of lymphocytes. Similar results were obtained with other intact cells, fibroblasts, hepatocytes and neuroblastoma cells using [^{3}H]spiperone and other ligands, such as [^{3}H]haloperidol, [^{3}H]pyrilamine and [^{3}H]ketanserin. Here again, displaceable binding was only present in intact cells but not in membrane fractions. Such a 'displaceable' binding was not related to receptor sites but may be regarded as non-specific binding which should correspond to a trapping phenomenon presumably in the lysosomes. Binding studies on intact cells need more caution than when performed on membrane preparations; indeed, permeation or trapping of ligands in the nanomolar range represents a serious drawback which, sometimes, can give the illusion of specific binding.

Most receptor binding studies are performed in membrane preparations and sometimes in soluble extracts. To characterize specific binding in such preparations, various conditions have to be considered: the choice of ^{3}H-labelled ligands and the displacer for non-displaceable binding (blank), the optimum binding conditions and areas of distribution in the brain or in different organs.

As a rule, binding characteristics on intact cells have been much less documented, although the use of cultured cells is becoming more and more popular in order to understand better the functional aspects and the internalization of receptor sites. Moreover, it is likely that peripheral blood cells are more easily accessible in man, either in normal or in pathological states. β-Adrenergic receptors have been demonstrated in human leucocytes [1–6]; although a good correlation was found between the binding in leucocytes and in other organs, the physiological meaning of such peripheral β-adrenergic receptors remains unclear. Other studies have shown binding sites labelled with muscarinic antagonists [7, 8]; however, the relative high K_D for the ^{3}H-labelled ligands and the very low potency of atropine and other drugs, especially when compared to their high affinity in brain homogenates, have allowed us to rule out the muscarinic nature of such binding sites [9]. Similarly, [^{3}H]dexetimide binding in lymphocytes did not reveal the characteristics of muscarinic receptors [10].

Recently, Le Fur *et al.* [11] reported the occurrence of [^{3}H]spiperone binding in human lymphocytes as being an index for the presence of dopamine receptors on these blood cells; moreover, they found marked alterations of these sites in a pathological state such as Parkinson disease [12, 13]. More recently, we and other groups demonstrated that [^{3}H]spiperone binding on human lymphocytes did not correspond to binding on dopamine receptors [10, 14, 15]. We suggested that such a displaceable binding could represent a trapping phenomenon presumably in the lysosomes [9].

In this paper, we report in more detail the [^{3}H]spiperone binding in lymphocytes; this preliminary observation was extended to other cell types using various ligands.

MATERIALS AND METHODS

Chemicals. [^{3}H]Spiperone (specific activity 20.0 Ci/mmole), [^{3}H]haloperidol (specific activity 22 Ci/mmole), [^{3}H]ketanserin (specific activity 22.3 Ci/mmole) were obtained from NEN (Boston, MA) and [^{3}H]pyrilamine (specific activity 24.1 Ci/mmole) was from Amersham (U.K.). Ficoll Paque was purchased from Pharmacia Fine Chemicals (Sweden). Dulbecco's modified Eagle's medium was purchased from Gibco Biocult Laboratories Ltd. (Scotland, U.K.).

Lymphocyte isolation. Fresh heparinized blood (40–100 ml) (heparine Novo 50 U/20 ml) was collected from healthy, non-medicated volunteers and diluted with one volume of Hank's balanced salt solution. Lymphocytes were isolated by centrifugation at 1000 *g* for 20 min of a Ficoll gradient according to the method of Boyum [16]. The band of lympho-

‡ To whom correspondence should be addressed.

cytes was collected, and after lysis of the contaminant erythrocytes with hypotonic solution, the cells were washed twice with Hank's buffer and passed through a nylon filter (26 μm). Cells were counted with a Coulter counter and diluted with buffer to obtain a final concentration of 1×10^6 cells/ml. More than 95% of the cells were viable as revealed by eosine coloration. The white cell composition showed an average of 95%lymphocytes with a small platelet contamination. Intact cells in suspension were used as such or homogenized (eight up-and-down strokes in a Potter homogenizer).

Cell culture. N1E115 neuroblastoma clone and the NH15CA2 hybrid cells were obtained from Dr. B. Hamprecht, Wurzburg, F.R.G.

The composition of the medium used for the N1E115 neuroblastoma cells was as follows: Dulbecco's modified Eagle's medium (DMEM) with 20 mM D-glucose and 10% foetal calf serum. The clone NH15CA2 was grown in the same DMEM medium but supplemented by 0.1 mM hypoxanthine, 1 μM aminopterin, 16 μM thymidine (DMEM-HAT) [17]. HEPES buffer had the following composition: NaCl 137 mM, KCl 5.4 mM, NaH_2PO_4 1 mM, D-glucose 5.5 mM, sucrose 20 mM, HEPES 20 mM, at pH 7.4.

Cells were maintained in DMEM or DMEM-HAT at 37° in a humidified atmosphere of 10% CO_2 and 90% air. Subcultures were made twice a week, 1:4 for N1E115 and 1:5 for NH15CA2. For binding assays, cells were washed three times with HEPES buffer and detached by shaking.

Binding assays. Intact lymphocytes (1×10^6 cells) were incubated without agitation in a total volume of 1.4 ml with 2 or 5 nM [^{3}H]spiperone or 2 nM of the other ligands. After incubation for 1 hr at 37°, cells were rapidly filtrated on GF/B Whatman filters and washed three times with ice-cold Hank's buffer. Filtration time did not exceed 10 sec. Then the filters were placed in plastic vials with 7 ml Insta-Gel (Packard) and counted for radioactivity in a Packard scintillation counter. All experiments were performed in triplicate. Throughout this paper, the term 'displaceable binding' is used instead of 'specific binding' to define the difference between total binding and blank (binding in the presence of 10^{-6} M haloperidol in [^{3}H]spiperone binding).

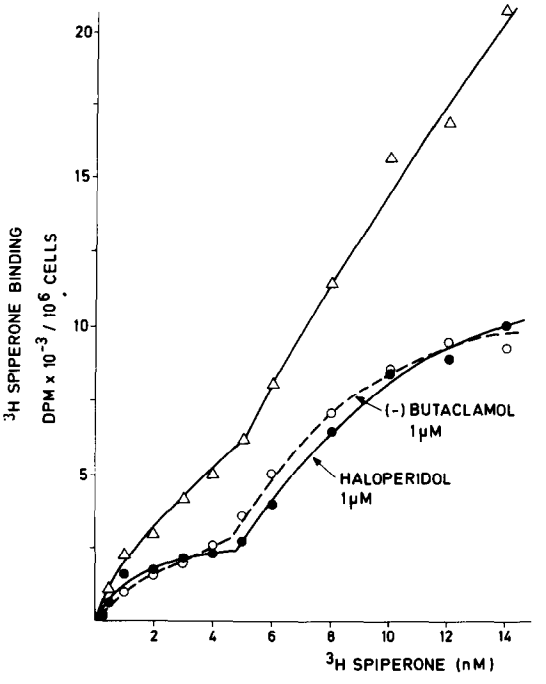

Fig. 1. Intact lymphocytes were incubated at 37° for 1 hr with increasing concentrations of [^{3}H]spiperone (0.2–14 nM). Non-displaceable binding is not represented in this figure, but it increases linearly in both cases. Points are mean values of three experiments each performed in triplicate. (△) Total binding, (●) displaceable binding obtained with haloperidol (1 μM), (○) displaceable binding obtained with (−)-butaclamol (1 μM).

Binding studies on neuroblastoma cells were performed in 2.4 ml of buffer, which was then incubated at 37° for 30 min or 1 hr. The filtration method used was the same as that for lymphocytes, except GF/C Whatman filters were used. Binding in membrane fractions was performed as previously described [18]. TCA-precipitable protein content was approximately 0.5 mg/sample in cell or membrane prep-

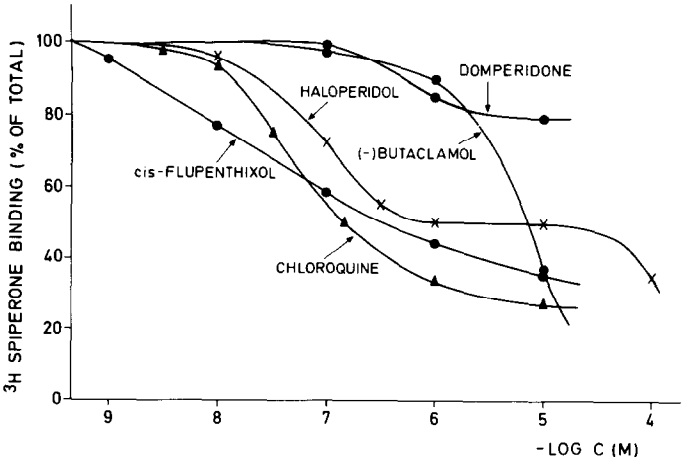

Fig. 2. [^{3}H]-Spiperone binding with different concentrations of cold drugs. Intact lymphocytes were incubated with [^{3}H]spiperone (5 nM). Each point is the average of two different experiments carried out in triplicate.

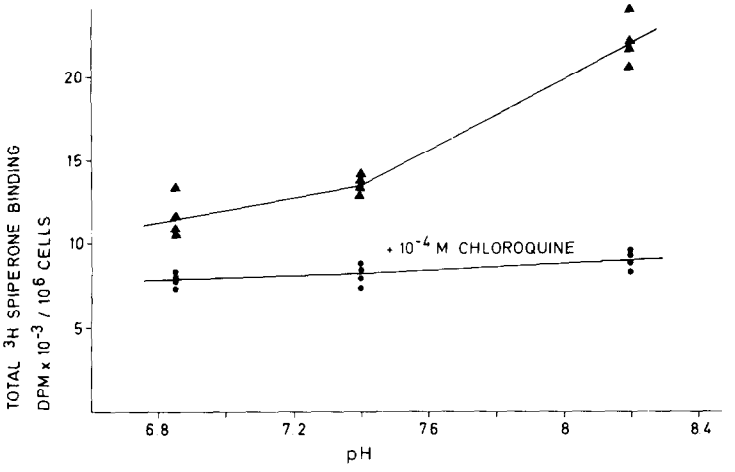

Fig. 3. [³H]Spiperone binding (8 nM) with lymphocytes at different pHs in isotonic buffer (▲). Binding was performed 30 min at 37° and the viability of cells was controlled before and after experiments. The same experiment was performed in the presence of chloroquine (10^{-4} M) (●).

arations and was determined by the Lowry method, with BSA as standard [19].

RESULTS

Binding on lymphocytes

Intact cells (10^6) were incubated with increasing concentrations of [³H]spiperone in the presence or absence of 10^{-6} M haloperidol. Figure 1 shows a biphasic curve with transient plateau at about 5 nM, followed by a second more linear component. Scatchard analysis was curvilinear and the K_D value of the high affinity component was 3.40 ± 0.7 nM (mean value of three experiments). On the contrary, the non-displaceable binding increased linearly. More surprisingly was the fact that when the pharmacologically-inactive enantiomer (−)-butaclamol (10^{-6} M) was used instead of haloperidol for the blank, the same displaceable binding was observed.

Figure 2 shows displacement curves obtained with several drugs which were tested in the [³H]spiperone binding on intact human lymphocytes. When haloperidol (10^{-6} M) was used to determine the blank value, displaceable binding represented 50–60% of the total binding. IC_{50} values markedly differed from those obtained in rat striatum (Table 1). The potent dopaminergic antagonist domperidone, for instance, was less active on lymphocytes than other drugs like chloroquine, alprenolol or opiate derivatives. Binding was maximal at 37° whereas at 4° only 65% of this binding was obtained. Total binding was greater at pH 8.2 than at 7.4 or 6.8 (Fig. 3). Such a dramatic pH dependence of buffer pH was not observed in rat brain (not shown here).

The association rate of [³H]spiperone in lymphocytes was slower than on dopaminergic receptors from striatal membranes, but the release of [³H]spiperone from the cells was extremely rapid. After incubation with 2 nM [³H]spiperone for 1 hr at 37°, the cells were diluted in 10 volumes of cold Hank's buffer and then rapidly centrifuged (10 min, 1000 *g*). 62% of the total spiperone bound was lost after the first washing, 86% after the second and 93% after the third. Such a rapid dissociation at low ligand concentrations did not allow us to study the subcellular distribution of the labelling.

In other experiments lymphocytes were incubated with cold drugs at relatively high concentrations and then assayed in the [³H]spiperone binding. Disappearance of the binding was observed after preincubation for 1 hr with chloroquine (10^{-4} M), alprenolol (10^{-5} M) and R5573 (opiate derivative 10^{-6} M). Displaceable binding also disappeared when the incubation was carried out in the presence of drugs

Table 1. Affinity of drugs to displace [³H]spiperone binding.

Drug	IC_{50} (nM)	
	Lymphocyte	Rat striatum
α-Flupenthixol	13	23
Chloroquine	31	7600
Spiperone	66	1.3
Haloperidol	84	20
R5573	110	>10,000
(+)-Butaclamol	1200	20
(+)-2-(*N*,*N*-Dipropyl)amino-5,6-dihydroxytetralin	2500	130
Ketanserin	2700	—
(−)-Butaclamol	4700	2100
Domperidone	>10,000	58

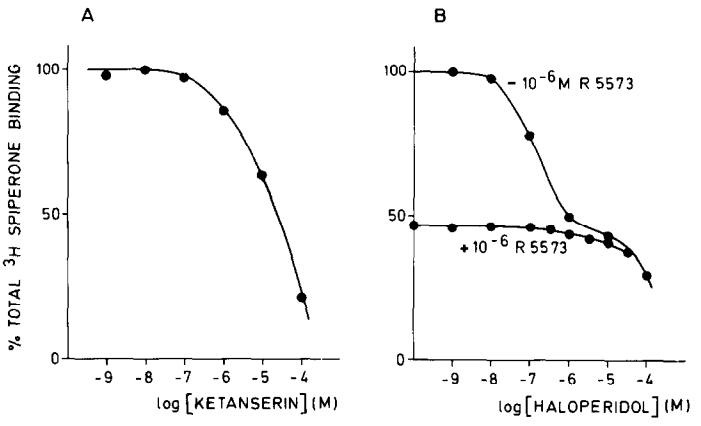

Fig. 4. (A) [^{3}H]Spiperone binding (5 nM) to intact lymphocytes was displaced by increasing concentrations of the serotonin S_2-antagonist ketanserin. (B) [^{3}H]Spiperone binding (5 nM) was performed in the absence or presence of 1 μM R5573. Haloperidol was used as displacer.

known to be inactive on the dopaminergic receptor.

Figure 4 shows the displacement of [^{3}H]spiperone by ketanserin, a potent serotonin S_2-antagonist, and by haloperidol in the presence and the absence of R5573 (10^{-6} M); with the latter, the remaining binding was no longer displaceable. When cells were incubated with L-dopa, L-tyrosine, L-methionine, valproate or acetyl salicyclic acid, an increase in [^{3}H]spiperone bound was observed (J.-M. Maloteaux, in preparation). Figure 5 shows that binding performed on membrane preparations revealed no specific component. Values of total binding were six times lower on membrane preparations (41 ± 16 fmole/10^{-6} cells) than on intact cells (238 ± 55 fmole/10^{-6} cells) using 5 nM [^{3}H]spiperone.

In addition to [^{3}H]spiperone, other ligands were tested; [^{3}H]haloperidol revealed a relatively high binding which was displaceable at high concentrations of various drugs. Figure 6 shows the displacement curve obtained with cold spiperone. The use of [^{3}H]pyrilamine at 2 nM revealed displaceable binding to intact lymphocytes for several antihistamine drugs (astemizole, clemastine, promethazine and pyrilamine)—but only at high concentrations, thus providing IC_{50} values higher than 10^{-7} M. With intact cells, alprenolol, a β-blocking agent displaced more [^{3}H]pyrilamine sites than pyrilamine itself.

Binding to neuroblastoma cells

Figure 7 shows the binding in intact N1E115 neuroblastoma cells with 2 nM [^{3}H]spiperone; cold spiperone was found to displace 50% of the total bound at 3×10^{-6} M, but chloroquine, as was the case with lymphocytes, was more potent. After homogenization of cells in hypotonic buffer, binding was performed in total homogenates or on total particulate fractions. There was no specific binding and the total binding was quite low. Identical observations were made when [^{3}H]haloperidol or [^{3}H]ketanserin was used as ligand; binding on intact cells was displaceable at high concentrations of cold drugs but there was no specific displacement on membrane preparations with (+)-butaclamol and methysergide,

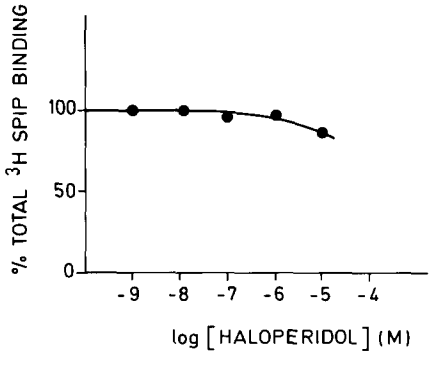

Fig. 5. Binding on the membrane preparation of lymphocytes. Human lymphocytes were isolated as described in Materials and Methods. Cells were homogenized and the membrane was diluted to obtain the final volume corresponding to 10^6 cells/ml. Binding was performed using 2 nM [^{3}H]spiperone and cold haloperidol as displacer. Very similar results were obtained 30 min and 1 hr after incubation. Points are the mean values of experiments carried out in triplicate.

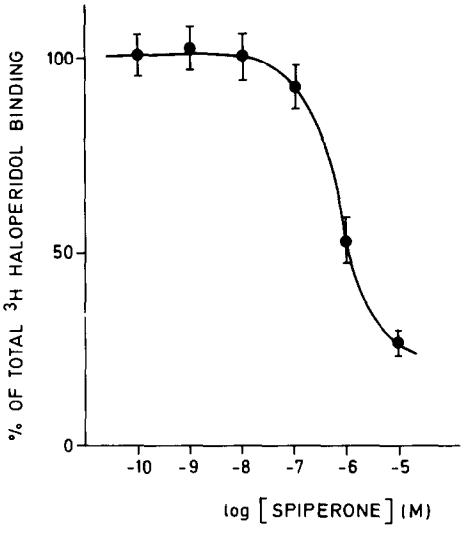

Fig. 6. Intact lymphocytes were incubated 30 min at 37° with [^{3}H]haloperidol (5 nM). Cold spiperone was used as displacer. Each point is the average of two separate experiments carried out in triplicate. Bars represent the S.D.

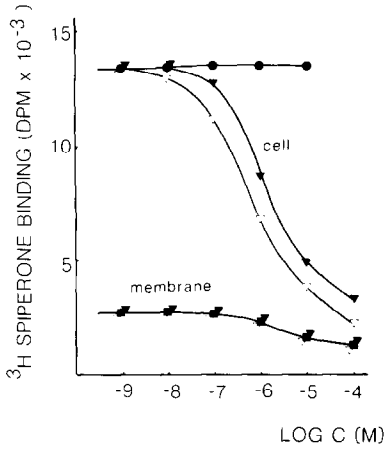

Fig. 7. N1E115 neuroblastoma cells were grown and detached as described in Materials and Methods. Binding was performed on intact cells in isotonic buffer with [3H]spiperone (2 nM) and increasing concentrations of cold spiperone (▼), chloroquine (▽) or L-dopa (●). The same amount of cells was used to obtain the membrane fraction (cf. Materials and Methods). Incubation of membrane fractions was performed in the presence of cold spiperone (▼), chloroquine (▽) or haloperidol (■). Each point represents the mean value of three determinations.

respectively. [3H]Pyrilamine binding was assayed in intact NH15CA2 cells; a large amount of [3H]pyrilamine was found to be bound and 50% was displaced by 1 μM cold pyrilamine. When the cells were homogenized in isotonic buffer with a Duall homogenizer, the binding was lower and the displacement occurred at the same range of high concentrations. Finally, when the cells were completely broken down by homogenizing in hypotonic Tris-buffer, the binding was markedly reduced and was no longer displaceable.

DISCUSSION

In an attempt to study [3H]spiperone binding in lymphocytes, binding properties of various ligands were characterized in these cells and then extended to other cellular types using different ligands. Binding properties were different according to which intact cells or membrane preparations were used in the [3H]spiperone binding assay. With lymphocytes we obtained saturation with [3H]spiperone in using either haloperidol or (+)- and (−)-butaclamol for the determination of the blank. Moreover, IC_{50} values of various drugs were not correlated to those obtained with rat striatum. Drugs inactive on dopaminergic receptors, such as chloroquine or R5573, were very potent in displacing [3H]spiperone in intact lymphocytes, whereas dopaminergic antagonists such as domperidone or sulpiride were practically inactive. Stereospecificity was present, though very weak. Therefore, [3H]spiperone did not seem to recognize dopamine receptors in lymphocytes.

In brain, in addition to the dopamine receptors, [3H]spiperone is known to bind with a high affinity on serotonin S_2-receptors in the frontal cortex [20]. Ketanserin displaced [3H]spiperone in lymphocytes giving an IC_{50} value of 2.7 μM, thus 100 times higher than in brain. Therefore, spiperone binding in lymphocytes was not of a serotoninergic nature.

In addition to its high affinity for D_2- and S_2-receptors, spiperone has also been reported to bind on spirodecanone sites [21, 22, 23]. In order to test this hypothesis, opiate drug R5573 [24], which binds with high affinity to spirodecanone sites [23, 25], was tested in intact lymphocytes; in the presence of this compound at 10^{-6} M, there was no longer displaceable binding with haloperidol. Hence this rules out the possibility that the binding did occur on spirodecanone sites.

Binding studies with other ligands showed that this non-specific phenomenon was not restricted to neuroleptic drugs; when using [3H]pyrilamine, a ligand for histamine H_1-receptors, binding occurred on the intact cells (lymphocytes or neuroblastoma cells) but was markedly reduced after cell homogenization. The same was observed when [3H]ketanserin, [3H]diazepam, [3H]alprenolol or [3H]astemizole (not shown here) was used with either intact lymphocytes or with neuroblastoma cells, neuroblastoma-glioma hybrid cells, fibroblasts or hepatocytes in culture.

These results indicate that the binding observed on intact cells in our experimental conditions was not related to specific binding on a true receptor. Evidence has been accumulated suggesting that this binding should correspond to a trapping in the cell. First, [3H]spiperone or [3H]pyrilamine binding was reduced and not displaceable when membrane preparations instead of whole cells were used. Secondly, the pH seemed to be more critical in intact cells than on membrane preparations, especially in the spiperone binding. Binding to lymphocytes was more pronounced at alkaline pH. Thirdly, chloroquine, which is known to be a lysosomotropic drug, was one of the most potent compounds to displace [3H]spiperone in lymphocytes and neuroblastoma cells; this suggests that chloroquine could prevent the accumulation of 3H-labelled ligands into the intracellular lysosomal compartment. Such a trapping phenomenon of ligands implies diffusion through biological membranes and concentration in lysosomes. This trapping is displaceable with weak base compounds of any chemical class without correlation to the pharmacological properties as described for several lysosomotropic agents [26], and it represents a serious pitfall when binding studies are performed on intact cells.

Figure 8 shows the different possibilities for 3H-labelled ligands to bind to intact cells. First, such a binding can reveal a receptor site; in this case all the criteria which make a binding site a receptor site are fulfilled [27]. As shown in Fig. 8, these receptors are located either on the cell surface or in synaptic vesicles. Secondly, binding can occur on acceptor sites or sites of recognition for a given ligand. The spirodecanone site labelled with [3H]spiperone is a good example of this. Thirdly, one can observe endocytosis mediated through a receptor or acceptor site; axonal transport of [3H]lofentanil in the vagus implies such an endocytosis of the 3H-labelled ligand bound to the opiate receptor [28].

In this paper we present a fourth possibility: the diffusion of labelled ligand through the cell mem-

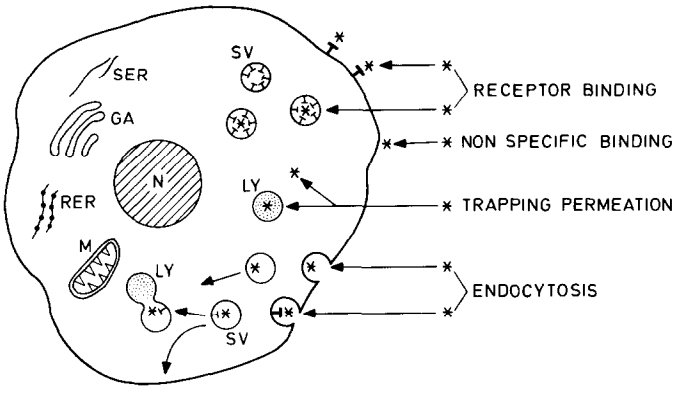

Fig. 8. Schematic representation of the possible interactions in intact cells of labelled ligands. RER: Rough endoplasmic reticulum; SER: smooth endoplasmic reticulum; GA: Golgi apparatus; M: mitochondria; LY: lysosomes; SV: synaptic vesicles.

brane and its accumulation in the lysosomal compartment. In the neuroblastoma cell, such a trapping phenomenon may be clearly differentiated from specific binding on muscarinic receptors (A. Gossuin, in preparation). Owing to such a trapping, more caution is needed when studying the binding of lipophilic ligands of low molecular weight on intact cells. This phenomenon is not restricted to psychotropic drugs but seems to be the common property of weak bases. It is probably a common phenomenon and lymphocytes seem more sensitive to the lysosomotropic agents [26, 29, 30].

Up to now, the pharmacological role of the trapping remains unclear; however, it could represent a storage site for some drugs and at much higher concentration it might be responsible for some toxic effects such as phospholipidosis [31, 32].

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