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Release Mechanisms of [^{125}I]Meta-iodobenzylguanidine in Neuroblastoma Cells: Evidence of a Carrier-mediated Efflux

T. Servidei, A. Iavarone, A. Lasorella, S. Mastrangelo and R. Riccardi

[^{131}I]metaiodobenzylguanidine ([^{131}I]MIBG) is selectively taken up and stored by tumours derived from the neural crest, and is used for diagnosis and treatment of neuroblastoma (NB). The antitumoral effect of [^{131}I]MIBG is closely related to the intracellular level of the radiopharmaceutical compound, which is dependent on uptake and storage/release mechanisms. While MIBG uptake is well characterised, storage and release mechanisms are still controversial. In order to better characterise [^{125}I]MIBG release mechanisms, we studied the basal and stimulated efflux of [^{125}I]MIBG in the human NB cell line, SH-SY5Y, preloaded with 0.1 μM [^{125}I]MIBG for 1 h. We found that [^{125}I]MIBG basal efflux is highly temperature-dependent, that [^{125}I]MIBG release, induced by cell depolarisation with high potassium, is mainly calcium-independent, and induced by exchange with cold MIBG or noradrenaline, inversion of the sodium gradient across the cell membrane by veratridine or by substitution of sodium chloride with equimolar concentration of lithium chloride. The exposure of NB cells to imipramine, an Uptake-1 inhibitor, also produces a net stimulatory effect on [^{125}I]MIBG release. However, when used in association with other releasing stimuli, such as higher levels of intracellular sodium or external agonists, imipramine abolishes the consequent increase of [^{125}I]MIBG release. Our findings suggest that stimulated [^{125}I]MIBG release is mediated by a carrier, most probably the uptake carrier working in a reverse mode, while a minimal fraction of [^{125}I]MIBG is released by an exocytotic mechanism.

Key words: neuroblastoma, cell lines, [^{131}I]metaiodobenzylguanidine ([^{131}I]MIBG), efflux, release, neurotransmitter, carrier-mediated

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INTRODUCTION

[^{131}I]METAIODOBENZYLGUANIDINE ([^{131}I]MIBG), a structural analogue of noradrenaline, is selectively taken up by neural crest derived cells, and is currently used in scintigraphic visualisation of neuroblastoma (NB) [1, 2]. [^{131}I]MIBG at high dosage is also employed in the treatment of disseminated NB with encouraging results [3, 4]. Theoretically, the cytotoxic effect of [^{131}I]MIBG may be improved either by stimulation of the uptake system or by some inhibition of the release mechanism, since both processes will result in increased intracellular levels and prolonged exposure to the radioactive iodine.

In competent cells, MIBG is mainly taken up by a specific uptake system (Uptake-1 system), which is sodium-, energy- and temperature-dependent and imipramine sensitive [5–7]. This specific uptake system involves the noradrenaline carrier, which physiologically brings about the reuptake of the neurotransmitter released at the synaptic cleft. Recently, the noradrenaline/MIBG transporter has been cloned, and the amino acid sequence predicted. This transporter shares a high homology with other neurotransmitter transporters [8].

Correspondence to R. Riccardi.
All the authors are in the Division of Pediatric Oncology, Catholic University, L.go A. Gemelli, 8–00168 Rome, Italy.

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While the molecular mechanisms underlying MIBG uptake in NB cells have been well elucidated in a number of *in vitro* studies [9–11], the mechanisms of MIBG retention and release remain poorly understood. The initial hypothesis supporting the retention of MIBG in neurosecretory granules and its secretion through exocytosis [12] has been replaced by the proposal that MIBG accumulation may depend on a dynamic equilibrium generated by the reuptake of the released drug [13].

Whereas this hypothesis is now widely accepted, it cannot explain some experimental observations, such as the heterogeneity of MIBG retention in NB cell lines which possess comparable specific uptake systems [9, 10]. Thus, the mechanism of MIBG retention/release appears to be a more complex phenomenon than previously reported.

It is tempting to hypothesise that not only MIBG uptake, but also MIBG release, may be mediated by a specific carrier. This mechanism would be similar to those reported for other amine transmitters, including noradrenaline, which have sodium-coupled carriers in the plasma membrane (for reviews see refs [14–16]). These carriers mediate unidirectional transport in the inward direction, according to the sodium gradient across the cell membrane. If depolarisation or the inversion of the sodium gradient favours transport in the outward direction, these carriers mediate substrate efflux by working in a reverse mode. The

neurotransmitter carriers can also mediate the simultaneous exchange of molecules of the same (homoexchange) or of similar substrates (heteroexchange) present on both sides of the cell membrane. In this case, the efflux of a radioactively labelled substrate can be accelerated by the presence of unlabelled substrate in the external medium (accelerated exchange diffusion) [17].

In order to evaluate if and to what extent MIBG release may be carrier-mediated, we studied the biochemical characteristics of basal and stimulated MIBG release in the NB cell line, SH-SY5Y.

MATERIALS AND METHODS

Chemicals

[^{125}I]MIBG (specific activity 3 m Ci/mg) and unlabelled MIBG were purchased from Amersham Buchler, Braunschweig, Germany. Imipramine, cold [-]-noradrenaline and veratridine were obtained from Sigma (St. Louis, Missouri, U.S.A.).

Cell culture

The human NB cell line, SH-SY5Y, the neuroblastic subclone of the cell line SK-N-SH, was a gift of Dr J.L. Biedler (Memorial Sloan-Kettering Cancer Center, New York, U.S.A.). The cells were grown in a 1:1 mixture of Eagle's minimal essential medium and Ham's F12, supplemented with nonessential amino acids, glutamine (2 mM) (Biowhittaker, Verviers, Belgium) and 15% heat inactivated fetal calf serum (Biological Industries, Israel) at 37°C in humidified 5% CO_2. All experiments were conducted in triplicate and results were expressed as mean ± standard deviation.

Temperature dependence of release

After 1 h incubation with 0.1 μM [^{125}I]MIBG, the supernatant was removed and the cells were washed twice to eliminate non-specific cell radioactivity. The cells were then exposed, for different time intervals up to 2 h, to medium without radiotracer, maintained at 4, 25 and 37°C. At the end of the releasing period, the medium was removed, the cells were washed twice with cold Hepes-buffered Krebs-Ringer solution (KRH) and lysed with 1.5 ml aliquots of 0.3 N sodium hydroxide. The radioactivity remaining in the cells was quantified in a γ-counter and expressed as the percentage of [^{125}I]MIBG accumulated at 1 h. Q_{10} (37°C/25°C efflux rate ratio) was calculated according to the method reported by Paton [18].

Stimulated [^{125}I]MIBG release

After preloading the cells for 1 h with 0.1 μM [^{125}I]MIBG, the cells were exposed to different stimulatory solutions up to 2 h. In some experiments, the stimuli were applied for a maximum of 30 min after 2 h of spontaneous release. The releasing medium was KRH solution modified according to the experimental strategy: in sodium-depleted medium, NaCl was replaced by an equimolar concentration of LiCl; for potassium-stimulated [^{125}I]MIBG release, the potassium concentration used was 56 mM and the sodium concentration was decreased in order to keep osmolarity constant. To study calcium-dependency, potassium-stimulated [^{125}I]MIBG release was studied in the presence of either 20 mM $MgCl_2$, to compete with calcium ion influx, or the calcium blocker, nitrendipine (1 μM).

Substances were used at the following concentrations: imipramine, 1 μM; veratridine, 100 μM; cold MIBG, 10 μM; cold noradrenaline, 50 μM; and added to releasing medium from 100-fold concentrated stock solutions.

RESULTS

Temperature dependence of [^{125}I]MIBG release

Basal [^{125}I]MIBG release was studied at 37, 25 and 4°C (Figure 1). Approximately 40% of [^{125}I]MIBG was released at 37°C after 2 h, without any appreciable additional efflux up to 24 h. When the temperature was lowered, the efflux was markedly reduced. Indeed, a reduction in temperature to 25°C caused an immediately marked fall in rate coefficient, with a Q_{10} of approximately 2.6 between 25°C and 37°C. At 4°C, only a minimal fraction (10%) of [^{125}I]MIBG was released after 2 h.

Stimulation of [^{125}I]MIBG release

The strict temperature dependence of [^{125}I]MIBG release prompted us to evaluate the involvement of a membrane system capable of transporting [^{125}I]MIBG out of the cell, similarly to the carriers for other amine transmitters. We therefore exposed [^{125}I]MIBG preloaded cells to different releasing stimuli, shown to activate carrier-mediated transport. The stimuli used were: 56 mM potassium (cell depolarisation); 10 μM cold MIBG (accelerated exchange diffusion), sodium-depleted medium (reversal of the uptake system by the inversion of the sodium gradient).

All three experimental conditions were efficient systems for the stimulation of [^{125}I]MIBG above the basal level (Figure 2). The rate of release was constant up to 60 min after uptake, when the fraction of [^{125}I]MIBG released was approximately double that of the control, and then it reached a plateau. The different stimuli appeared to have a similar effect on the induction of [^{125}I]MIBG release.

Saturability of [^{125}I]MIBG release

Previous results raised the possibility that the different releasing stimuli might be mediated by a common molecular system. To test this hypothesis, we exposed [^{125}I]MIBG preloaded cells to several different combinations of releasing stimuli (Figure 3). Regardless of the specific association used (Figure 3a, high potassium plus cold MIBG; Figure 3b, sodium-depleted medium plus cold MIBG; Figure 3c, high potassium plus sodium-depleted medium), the second stimulus was never able to increase release over the basal level of [^{125}I]MIBG release.

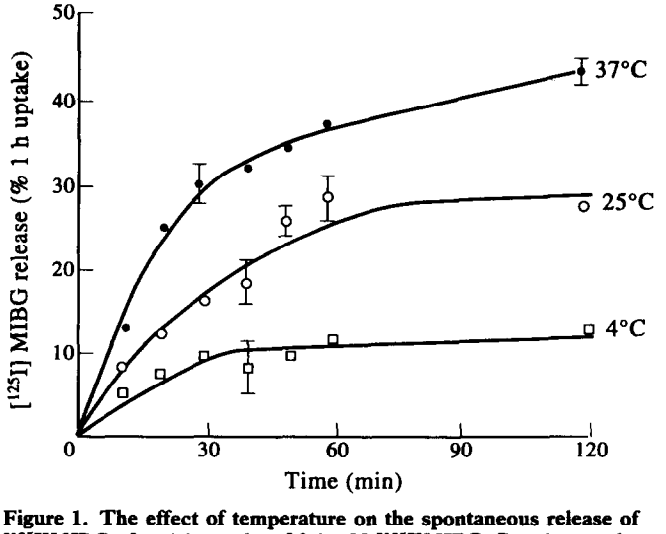

Figure 1. The effect of temperature on the spontaneous release of [^{125}I]MIBG after 1 h uptake of 0.1 μM [^{125}I]MIBG. Results are the means of triplicate values; bars, SD. SD is not shown when it does not exceed the symbol size.

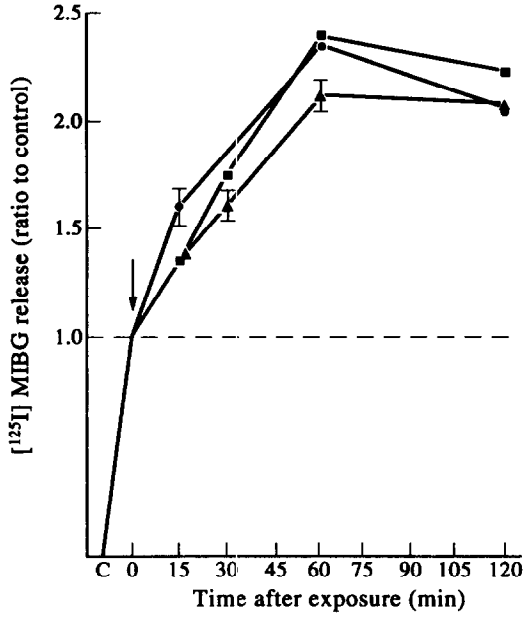

Figure 2. Effect of 10 μM cold MIBG (▲), zero-sodium (■) and 56 mM K^+ (●) on [125I]MIBG release. After preloading cells with 0.1 μM [125I]MIBG for 1 h, the cells were exposed to the stimuli, and [125I]MIBG release was determined at various time points. Results are expressed as the relative ratio of each stimulus measured under control conditions. Results are the means of triplicate values; bars, SD. SD is not shown when it does not exceed the symbol size.

This is consistent with a saturable mechanism responsible for [125I]MIBG release in this cell line. The mechanism can be activated and saturated by one stimulus, and cannot be additionally induced following the application of a different stimulus.

Effect of inhibitor of the Uptake-1 system on [125I]MIBG release

From an analogy with other amine carriers, the membrane system capable of transporting [125I]MIBG out of the cell could be the uptake carrier working in a reverse mode. If this were the case, then the competitive inhibitor of the uptake system, imipramine, should inhibit carrier-mediated [125I]MIBG efflux [17]. Imipramine binds the transporter protein but, because it does not undergo transport, the carrier does not regain mobility upon binding the ligand and is arrested outside the cell membrane. We tested the effect of imipramine upon [125I]MIBG release stimulated by high concentrations of carrier ligands (50 μM cold noradrenaline and 10 μM cold MIBG) and by the reversal of the sodium gradient with veratridine (100 μM), which opens the voltage-dependent Na^+ channels and causes Na^+ influx down its concentration gradient. After 2 h of spontaneous release, in the presence of a constant rate of efflux, the addition of imipramine (1 μM) to the medium increased the efflux by approximately 20% (Table 1). This increase in [125I]MIBG release may be a consequence of reuptake inhibition. However, imipramine was able to strongly reduce or almost abolish the stimulatory effect of cold MIBG, cold noradrenaline and veratridine. Interestingly, the inhibitory effect of imipramine was reduced when the stimuli were applied immediately after preloading cells (data not shown). At the concentrations of cold MIBG and cold noradrenaline used in these experiments, imipramine did not reduce their uptake; therefore, the inhibition of stimulated [125I]MIBG efflux by imipramine observed after 2 h of spontaneous release is probably a consequence of the immobilisation of the carrier outside the cell membrane, which makes it unavailable on the inside of the membrane to mediate outward transport.

Table 1. Effect of imipramine on evoked [125I]MIBG release

	% Increase [125I]MIBG release*	
	Experiment 1	Experiment 2
Noradrenaline	30.6 ± 4.3	40.1 ± 5.8
MIBG	17.5 ± 2.4	34.8 ± 2.3
Veratridine	14.3 ± 1.7	26.1 ± 2.4
Imipramine	19.1 ± 3.4	24.5 ± 4.8
Noradrenaline + imipramine	17.8 ± 5.5	20.1 ± 2.9
MIBG + imipramine	5.2 ± 3.6	14.2 ± 4.2
Veratridine + imipramine	2.0 ± 2.7	12.4 ± 2.5

* % Increase of [125I]MIBG released during 30 min of exposure to the indicated substances after 1 h uptake (0.1 μM [125I]MIBG) and 2 h of basal release (0% = [125I]MIBG released after 2 h and 30 min without stimulus). Concentrations used: imipramine = 1 μM; cold noradrenaline = 50 μM; cold MIBG 10 μM; veratridine = 100 μM.

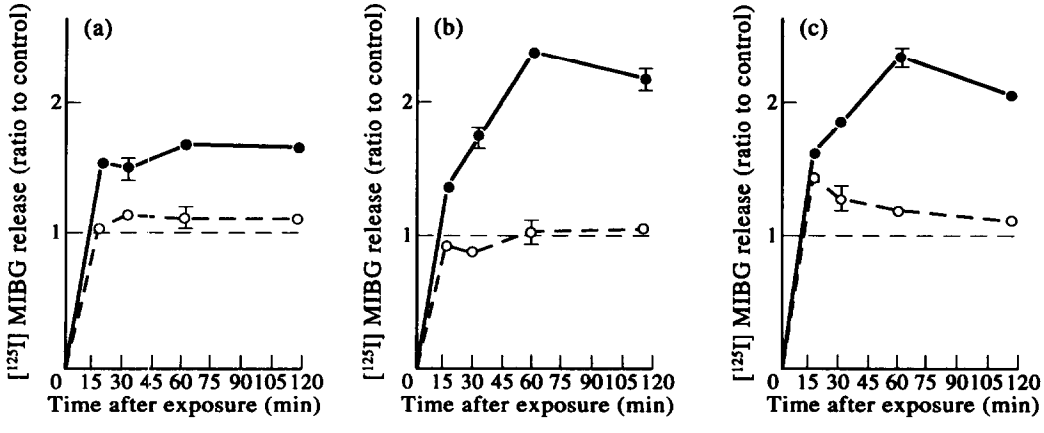

Figure 3. Effect of: (a) 56 mM potassium on basal (●) and 10 μM cold MIBG stimulated (○) [125I]MIBG release; (b) sodium depletion on basal (●) and 10 μM cold MIBG stimulated (○) [125I]MIBG release; (c) 56 mM potassium on basal (●) and sodium-depleted evoked (○) [125I]MIBG release. Cells were preloaded for 1 h with 0.1 μM [125I]MIBG and exposed for various times to one stimulus (●) or to a combination of two different stimuli (○). Results are expressed as the ratio of basal or evoked [125I]MIBG release. Results are the means of triplicate values; bars, SD. SD is not shown when it does not exceed the symbol size.

Calcium independence of [^{125}I]MIBG release

Intracellular pools of amino acid neurotransmitters have been related to two basic compartments: a vesicular pool, released via a Ca^{2+}-dependent exocytosis, and a cytoplasmic pool, released in a Ca^{2+}-independent manner, probably by the reversal of the uptake system [14–16]. To test the possible involvement of neurosecretory granules in potassium-stimulated [^{125}I]MIBG release, we determined the potassium-stimulated [^{125}I]MIBG release in the presence of high Mg^{2+} concentrations (20 mM) to compete for Ca^{2+} influx, or in the presence of nitrendipine, a known Ca^{2+} channel blocker (Figure 4). Even though, in both conditions, there was some inhibition of [^{125}I]MIBG release that proved to be significant, the fraction of Ca^{2+}-dependent [^{125}I]MIBG release was never higher than 15% when compared with the total potassium-stimulated [^{125}I]MIBG release.

DISCUSSION

In NB, both scintigraphy and targeted radiotherapy with [^{131}I]MIBG are related to the interrelationship between drug uptake and retention/release mechanisms, which control the intracellular levels and the exposure of cells to the radioactive iodine. Neuronal differentiation, pharmacologically induced in NB cells *in vitro*, is able to increase MIBG uptake [9], but the clinical relevance of this observation has not yet been proved. An alternative method of increasing the intracellular level of [^{131}I]MIBG would be to inhibit release mechanisms.

In order to evaluate whether [^{131}I]MIBG release in NB cells is susceptible to pharmacological modulations aimed at increasing [^{131}I]MIBG retention, we examined basal and stimulated [^{125}I]MIBG release in the NB cell line, SH–SY5Y.

The basal release of [^{125}I]MIBG appeared to be highly temperature-sensitive, with a Q_{10} between 25 and 37°C of about 2.6. Paton [18] reported a similar value (Q_{10} 2.5–3.0) for noradrenaline efflux from adrenergic nerves. This value may be compatible with a carrier-mediated process rather than a simple passive diffusion [17].

In neuronal systems, two distinct mechanisms of neurotransmitter release have been described: a Ca^{2+}-dependent release, corresponding to an exocytotic process, which involves the neurotransmitter pool stored in neurogranules; and a Ca^{2+}-independent release, which involves the cytoplasmic neurotransmitter pool and is mediated by the reversal of the uptake system. A carrier-mediated efflux has been described for a number of neurotransmitters (γ-aminobutyric acid, glutamate, dopamine, noradrenaline), which have Na^+ coupled transporters in the plasma membrane [14–16, 19–22]. The carrier-mediated release can be triggered by different stimuli, such as depolarisation, inversion of the Na^+ gradient across the cell membrane and trans-stimulation by high concentrations of carrier ligands: this last process, also termed accelerated exchange diffusion, has been reported in neuronal and non neuronal systems [23–25].

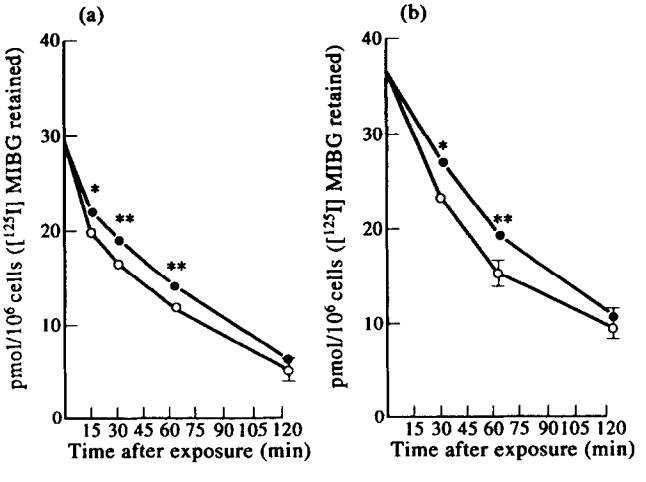

Figure 4. [^{125}I]MIBG retained after stimulation with: (a) 56 mM K^+ (○) or 56 mM K^+ plus 20 mM Mg^{2+} (●); (b) 56 mM K^+ (○) or 56 mM K^+ plus 1 μM nitrendipine (●). Results are the mean of triplicate values; bars, SD. SD is not shown when it does not exceed the symbol size. Statistical differences were analysed by Student's *t*-test: *$P < 0.05$; **$P < 0.01$.

When applied to the NB cells, SH–SY5Y, preloaded with [^{125}I]MIBG, all these stimuli (depolarisation, inversion of the Na^+ gradient and trans-stimulation) were capable of inducing [^{125}I]MIBG release to a similar extent above the basal level. Furthermore, the combination of different stimuli, regardless of the specific association used, did not exert an additive effect on [^{125}I]MIBG release evoked by a single stimulus. Taken together, these results suggest that a common molecular mechanism, probably the uptake system working in a reverse mode, underlies the stimulated [^{125}I]MIBG release. This mechanism can be activated and saturated by one stimulus, but, once activated to saturation, further stimulation by other means is ineffective.

Although reminiscent of a carrier-mediated mechanism for [^{125}I]MIBG efflux, these data do not exclude the possibility that the stimulation of [^{125}I]MIBG release, observed when Na^+ was replaced by lithium in the external medium, might be a consequence of inhibition of [^{125}I]MIBG uptake, which is Na^+-dependent. Similarly the increased [^{125}I]MIBG efflux following high concentrations of cold carrier ligands (MIBG or noradrenaline) could be related to a competitive inhibition of hot/cold substrate.

However, unequivocal proof of a carrier-mediated efflux for [^{125}I]MIBG comes from the inhibition of stimulated [^{125}I]MIBG release by imipramine, a competitive inhibitor of the uptake system. Indeed, if [^{125}I]MIBG release is mediated by its transport system, the imipramine is expected to antagonise that process [17, 26]. In our experimental system, imipramine strongly reduced and almost abolished the carrier-mediated release of [^{125}I]MIBG induced by homoexchange and heteroexchange with cold MIBG and cold norepinephrine, respectively, or by the reversal of the sodium gradient by veratridine. The observed inhibition of [^{125}I]MIBG efflux by imipramine may be a consequence of the immobilisation of the transporter outside the cell membrane, making it unavailable within the membrane to mediate transport in the outward direction.

Potassium-stimulated [^{125}I]MIBG release is mainly Ca^{2+}-independent: in the presence of the Ca^{2+} antagonist, nitrendipine, or of a high concentration of Mg^{2+}, [^{125}I]MIBG release was only slightly reduced. As reported by other authors [27, 28], our data also show that only a minimal fraction of [^{125}I]MIBG, probably stored in neurogranules, can be released in a Ca^{2+}-dependent manner through an exocytotic process. These observations are compatible with the presence of a small amount of neurosecretory granules in NB cells.

Although different mechanisms, such as exocytosis, carrier mediated-efflux and passive diffusion appear to underlie [^{125}I]MIBG release (Figure 5), the contribution by each of them could vary with the experimental conditions used (basal versus stimulated [^{125}I]MIBG release) and/or intracellular [^{125}I]MIBG levels. Since there is general agreement on the minor role of

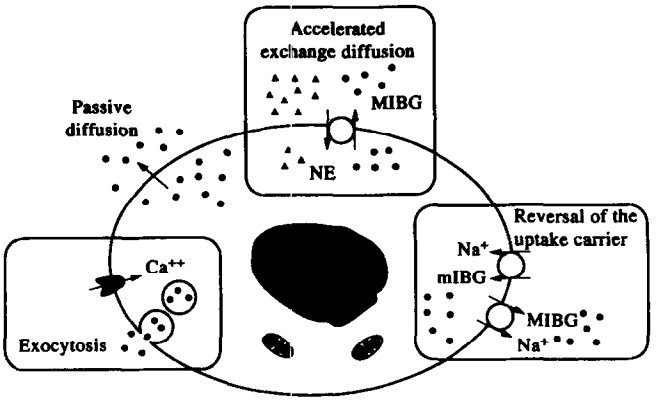

Figure 5. Schematic representation of the mechanisms probably involved in [125I]MIBG release. Along with a passive diffusion phenomenon and exocytosis, [125I]MIBG may be released by the uptake carrier working in a reverse mode. The latter mechanism can be triggered either by the inversion of the sodium gradient across the cell membrane or by trans-stimulation by a ligand outside the cell membrane. For further details see text. ● MIBG; ▲ noradrenaline.

exocytosis in [125I]MIBG release, the main release mechanisms could be carrier-mediated efflux and passive diffusion. According to the model proposed by Trendelenburg [17] for noradrenaline, the structural analogue of MIBG, it is expected that [125I]MIBG efflux mediated by a saturable carrier predominates at low, and efflux by passive diffusion at high, intracellular [125I]MIBG concentrations. Indeed, the inhibitory effect of imipramine on [125I]MIBG-stimulated release, which interferes with the carrier-mediated mechanism, was strongly reduced if the stimuli were applied immediately after preloading cells, when the intracellular [125I]MIBG concentration is very high (data not shown).

There is growing evidence that neurotransmitters can be released not only by exocytosis, but also through the membrane carriers responsible for transmitter reuptake [29]. A carrier-mediated neurotransmitter release, stimulated by similar experimental procedures, has been reported by many authors, in different model systems, ranging from synaptosomes, to neuronal and glial cell cultures, including NB cell lines, and brain slices [16–30]. Although this is the first report of a carrier-mediated release of [125I]MIBG from NB, it is conceivable that mechanisms operating for the efflux of noradrenaline, which shares the same carrier as [125I]MIBG, can be involved in [125I]MIBG efflux.

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