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Gene transfer of a reserpine-sensitive mechanism of resistance to *N*-methyl-4-phenylpyridinium

(vesicular amine transporter/Parkinson disease)

Y. LIU, A. ROGHANI, AND R. H. EDWARDS*

Department of Neurology, University of California at Los Angeles School of Medicine, 710 Westwood Plaza, Los Angeles, CA 90024-1769

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ABSTRACT **The toxin *N*-methyl-1,2,3,6-tetrahydropyridine produces a model of neural degeneration very similar to idiopathic Parkinson disease. To understand the cellular mechanisms that modulate susceptibility to its active metabolite *N*-methyl-4-phenylpyridinium (MPP^+), we have transfected a cDNA expression library from the relatively MPP^+-resistant rat pheochromocytoma PC12 cells into MPP^+-sensitive Chinese hamster ovary (CHO) fibroblasts. Selection of the stable transformants in high concentrations of MPP^+ has yielded a clone extremely resistant to the toxin. Reserpine reverses the resistance to MPP^+, suggesting that a transport activity protects against this form of toxicity, perhaps by sequestering the toxin within an intracellular compartment. In support of this hypothesis, dopamine loaded into the CHO transformant shows a localized distribution that is distinct from the pattern observed in wild-type cells and is also reversed by reserpine.**

Systemic administration of *N*-methyl-1,2,3,6-tetrahydropyridine (MPTP) results in the death of dopaminergic neurons in the substantia nigra (1). Since MPTP reproduces the pattern of neuronal degeneration observed in Parkinson disease (PD), this form of toxicity has been used as a model system to dissect the cellular components responsible for selective neuronal vulnerability in the idiopathic human disease. Toxicity requires conversion of MPTP to the active metabolite *N*-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B (2–4). Dopaminergic cells of the substantia nigra then accumulate high levels of MPP^+ by uptake through the high-affinity plasma membrane catecholamine transporter (5, 6). Inside the cell, MPP^+ enters mitochondria (7) and inhibits respiration at the level of complex I, apparently by binding near the site of action of the other mitochondrial toxins rotenone and piericidin (8, 9).

Studies of patients with PD support the utility of MPTP as a model for idiopathic PD. Both brain tissue and circulating platelets from patients with PD show a selective reduction in the same mitochondrial component affected by MPTP, respiratory complex I (10–12). Deprenyl, which inhibits the enzyme that activates MPTP, monoamine oxidase, also appears to slow the rate of progression in idiopathic PD (13). However, parkinsonism induced by MPTP develops over days to weeks, whereas idiopathic PD develops over years. Explanations for the relatively slow rate of progression in idiopathic PD include chronic, low levels of exposure to an environmental toxin similar to MPTP, oxidative stress related to the cytoplasmic accumulation of dopamine, or the trapping of free radicals by deposited dopamine and lipofuscin that predisposes these cells to mitochondrial injury (14).

The MPTP model has suggested multiple pathogenetic factors that may also contribute to idiopathic PD. The amelioration of toxicity with an excitatory amino acid antagonist implies that neural excitation plays a role in the injury produced by MPP^+ *in vivo* (15). In addition, neurotrophic factors such as brain-derived neurotrophic factor can protect midbrain cells from MPP^+ *in vitro* (16). However, accumulation of the toxin through a high-affinity plasma membrane catecholamine transporter is required for susceptibility to MPTP *in vivo*. The expression of this transport activity could account for the selective cell vulnerability observed in both the MPTP model and idiopathic PD, with several notable exceptions.

Adrenal chromaffin cells and postganglionic sympathetic neurons express a high-affinity catecholamine uptake system but, unlike dopaminergic neurons in the midbrain, do not degenerate either in response to MPTP or in most cases of idiopathic PD. Chromaffin cells have even been demonstrated to accumulate [^{3}H]MPP^+ but with relatively little toxicity (17). Rat pheochromocytoma PC12 cells derive from the adrenal gland and, although they have served as a model system to understand MPP^+ toxicity, PC12 cells show little susceptibility to the toxin, with 1 mM MPP^+ resulting in cell death by 4 days and with 100 μM MPP^+ resulting in death by 2 weeks (18, 19). Furthermore, inhibition of high-affinity plasma membrane uptake by tricyclic antidepressant drugs protects PC12 cells entirely from MPP^+, indicating that without an active system for its accumulation, these cells are intrinsically resistant to the effects of the toxin (6). Thus, the resistance of PC12 cells to MPP^+ toxicity can be used to dissect the differential MPP^+ susceptibility of aminergic populations in the midbrain, adrenal gland, and sympathetic ganglia.

Chinese hamster ovary (CHO) cells lack several neuronal features known to affect susceptibility to MPP^+, such as excitability, catecholamine uptake activity, and the receptors for neurotrophic factors. However, at high density, CHO cells have an extremely steep dose–response curve to MPP^+, with virtually complete inhibition of protein synthesis after exposure to >100 μM for only 2–3 days. (At low density, CHO cells show a similar threshold of sensitivity but simply stop growing until detachment from the plate days to weeks later.) Although they lack a plasma membrane catecholamine transporter and so have less sensitivity to MPP^+ than dopaminergic neurons *in vivo*, CHO cells still show greater sensitivity than PC12 cells to MPP^+. Thus, CHO cells can be used as a simplified system in which to identify the properties that confer resistance to this form of toxicity in PC12 cells, an aminergic line that actively accumulates MPP^+ but that, similar to the cells in the adrenal medulla from which it derived, shows little susceptibility to the toxin.

MATERIALS AND METHODS

cDNA Library Construction. The first strand of cDNA was synthesized from 5 μg of PC12 cell poly(A)$^+$ RNA (20, 21)

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Abbreviations: MPP^+, *N*-methyl-4-phenylpyridinium; MPTP, *N*-methyl-1,2,3,6-tetrahydropyridine; PD, Parkinson disease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

*To whom reprint requests should be addressed.

using oligo(dT) as primer and avian myeloblastosis virus reverse transcriptase (Life Sciences). The second strand was synthesized with RNase H and *Escherichia coli* DNA polymerase, and *Bst*XI linkers were added (22, 23). Free linker and short cDNA fragments were removed by electrophoresis through 5% acrylamide followed by electroelution of cDNA >1.5 kilobases. The cDNA was then ligated into the CDM8 vector and transformed into bacteria by electroporation (24).

Gene Transfer. Library plasmid DNA was coprecipitated with the Rous sarcoma virus–neo plasmid and transfected by a modified calcium phosphate procedure with the buffer BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] (Calbiochem) at pH 6.95 and 3% CO_2 (25).

MTT Assay. MPP^+ was added to wild-type and resistant cells at ≈50% confluence, and toxicity was assayed after 2–3 days by measuring general cell dehydrogenase activity with the reduction of the tetrazolium dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (26).

Oxygen Consumption. Respiration was measured in Krebs–Ringer buffer using a Clark oxygen electrode (Yellow Springs Instruments) (19).

Catecholamine Uptake. Uptake and cellular compartmentalization of loaded dopamine were performed by growing cells on polylysine-coated glass coverslips. After cell attachment, the medium was replaced with standard medium plus serum that contains 0.5–1.0 mM dopamine and was incubated for an additional 12–24 hr. The cells were then washed three times in 0.1 M sodium phosphate (pH 7.4) and incubated at 4°C in 2% glyoxylic acid/0.5 mg of $MgCl_2$ per dl, pH 4.9–5.0 for 3 min (27). The coverslips were then drained thoroughly, dried in air at 45°C, heated to 80°C for 5 min, inverted over mineral oil on glass slides, and examined under fluorescence with the appropriate filters (28).

RESULTS

To determine whether the transfer of sequences from PC12 cells into CHO cells could be used to generate stable CHO transformants resistant to MPP^+, it was first important to determine the spontaneous rate of resistance under different culture conditions. Untransfected CHO cells selected in 500 μM MPP^+ show different patterns of toxicity depending on cell density. At high density (>75% confluence), all the cells die within 24 hr, with no possibility for subsequent growth. At low density (<25% confluence), the cells stop growing, gradually acquire refractile cytoplasmic inclusions, swell, and eventually detach from the plate after several weeks. Selection of 10^6 CHO cells at intermediate density (25–50% confluence) in 500 μM MPP^+ gives rise to 5–10 small colonies after 1 month. However, these cells contain the particulate inclusions characteristic of MPP^+ toxicity and grow very slowly even when maintained at low density. At moderate density, the cells degenerate further and detach from the plate. Thus, untransfected CHO cells acquire little resistance to 500 μM MPP^+ during selection for >2 months. In addition, transfection with a selectable marker alone has yielded no clones significantly resistant to MPP^+.

Using an efficient, modified calcium phosphate procedure (25), we have transfected a PC12 cDNA library constructed in the plasmid expression vector CDM8 (23) into CHO cells, yielding 200–500,000 independent stable transformants. These cells were then selected at 40% confluence in 1 mM MPP^+. Virtually all the cells developed toxicity within 1 week, and many detached from the plate by 3 weeks, with no evidence of healthy cells at that time. However, at 4 weeks, a colony of cells without refractile inclusions appeared and rapidly covered the plate. The apparently normal growth of these cells in the presence of 1 mM MPP^+ stands in marked contrast to all of the resistant clones obtained without transfection, which grew very slowly if at all. Using the reduction of a tetrazolium dye (MTT assay) to measure cell toxicity (26), Fig. 1*a* shows the steep dose–response curve to MPP^+ in wild-type cells and the relative lack of toxicity in the selected resistant cell clone. However, at 1 mM MPP^+, the resistant cells do show some toxicity, particularly when the assay is done at higher cell density.

Since changes in cell density and hence presumably growth rate have been observed to affect MPP^+ toxicity, it was important to determine whether the transfected clone's resistance derived simply from a change in growth rate. However, repeated determination of growth rate in the resistant clone in the absence of MPP^+ showed no difference from wild-type CHO cells (data not shown). (In the presence of MPP^+, the resistant clone does grow slightly more slowly than untreated wild-type cells, particularly at higher densities.)

Resistance to MPP^+ could derive from improved ability of the cell to compensate for inhibition of respiration, such as by an increased dependence on glycolysis (19, 29). To determine the role of such compensatory mechanisms, we have used oxygen consumption to examine the primary site of MPP^+ action in the respiratory chain. If a compensatory mechanism were responsible for resistance, MPP^+ would be expected to inhibit oxygen consumption in both wild-type and resistant cells. As shown in Fig. 2*a*, 500 μM MPP^+ dramatically inhibits oxygen consumption by 12 hr after exposure in wild-type cells. However, in the resistant transfected cells, MPP^+ does not detectably affect oxygen consumption (Fig. 2*b*) Thus, although improved ability to compensate for respiratory injury remains a possible, additional mechanism for resistance to MPP^+, the principal mechanism for resistance in the clone appears to reside either in the primary site of action of the drug in the respiratory chain or in its metabolism and distribution by the cell.

To determine whether an alteration in the process of respiration could underlie the resistance to MPP^+, we studied the effects of other known respiratory inhibitors. Oligomycin inhibits complex III of the respiratory chain and shows the same pattern of toxicity in wild-type and in resistant transfected CHO cells (data not shown). This is consistent with the previous finding that the mechanism of resistance in these

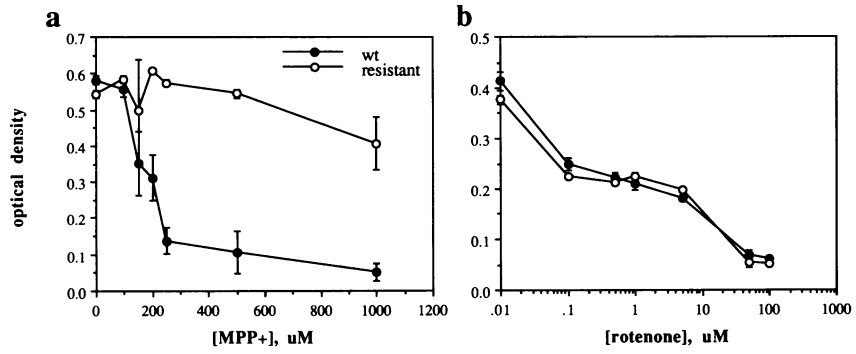

FIG. 1. Differential susceptibility to MPP^+ and rotenone. Wild-type (●) and MPP^+-resistant (○) CHO cells were grown for 2 days in the presence of the indicated concentrations of MPP^+ (*a*) and rotenone (*b*), and toxicity was determined by measuring general cell dehydrogenase activity with reduction of the tetrazolium dye MTT in a spectrophotometric plate assay. MPP^+-selected cells show marked resistance to the toxin but no difference from wild-type cells in susceptibility to rotenone. All determinations were performed in triplicate (mean ± SD).

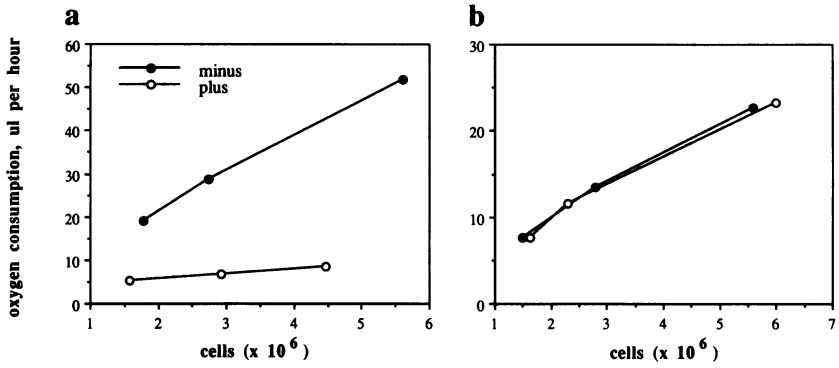

FIG. 2. MPP+ does not inhibit oxygen consumption in the MPP+-resistant CHO transfectant. Wild-type (*a*) and MPP+-resistant (*b*) cells were treated for 12 hr in 500 μM MPP+ and oxygen consumption was measured with a Clark electrode.

cells does not derive from an improved ability to compensate for respiratory injury. Rotenone inhibits complex I of the respiratory chain and competes with MPP+ for binding to its presumed site of action (8, 9). Thus, if the resistance were due to a change in the site of action, the transfected cells should also show resistance to rotenone. Repeated experiments have demonstrated no substantial resistance to rotenone in the transfectant (Fig. 1*b*). However, rotenone is considered to have its selective effect on respiration at relatively low doses and an additional, less specific effect at higher doses. Toxicity using the MTT assay does show a biphasic pattern in CHO cells (Fig. 1*b*), but in no dose range is there a clear difference of the resistant cells from wild type. Since the primary site of drug action is unaltered by these criteria, the mechanism of resistance in the transfected cells appears somewhat specific for MPP+, suggesting a role for altered drug metabolism or distribution.

CHO cells lack a system for the high-affinity uptake of catecholamines (data not shown), so resistance to MPP+ cannot be due to loss of this system. On the other hand, a different type of activity could be responsible for drug export. However, the uptake of 25 nM [^{3}H]MPP+ over 1–48 hr at 37°C showed no difference between wild-type and resistant cells (data not shown), providing no support for an active efflux mechanism. Thus, the cells show little evidence for an alteration in the primary site of drug action or for active export.

Previous studies have suggested a potential mechanism of resistance to MPP+ related to changes in cellular drug distribution. Fractionation of bovine adrenal chromaffin cells exposed to [^{3}H]MPP+ has shown that a large proportion of the radiolabeled material resides in chromaffin granules, with only small amounts in mitochondria (17, 30). Inhibition of the chromaffin granule amine transporter with tetrabenazine enhances the depletion of amines and reduction in tyrosine hydroxylase produced by MPP+, suggesting that this uptake mechanism might protect against the toxin by sequestering it in the granules. Since PC12 cells express reserpine-sensitive vesicular uptake of amines, it appeared possible that transfer of this activity was responsible for the resistance to MPP+ toxicity observed in the CHO transfectant. For these reasons, we examined the toxicity of MPP+ on the transfected cells in the presence of 1 μM reserpine and found dramatic reversion to wild-type CHO sensitivity (Fig. 3*a*). To demonstrate that reserpine does not affect a mechanism present in wild-type CHO cells, we have tested its effect on wild-type cells treated with the lower concentrations of MPP+ to which they are normally sensitive (Fig. 3*b*). The only changes occur at the top of the steepest section of the dose–response curve, which typically shows the greatest random fluctuation and standard deviation in this assay (Fig. 1). Thus, reserpine shows very little reproducible effect on MPP+ toxicity in wild-type cells. The results suggest that the transfected cell clone survives selection in MPP+ because the cells express a vesicular amine uptake activity, presumably derived from PC12 cells, that effectively sequesters the toxin from its primary site of action in mitochondria.

To observe directly whether the resistant cells express an intracellular amine transport activity, it was necessary to circumvent the absence of a high-affinity plasma membrane transport system in the CHO transfectant. Neuronal cells that synthesize amines usually express both plasma membrane and synaptic vesicle catecholamine transport activity. Detection of specific uptake requires amines radiolabeled to high specific activity. Using either labeled MPP+ or catecholamine in the 10–100 nM range, it is possible to detect both specific plasma membrane and vesicular uptake in neuronal cultures. However, if the cell lacks a high-affinity plasma membrane transporter, catecholamines at these low concentrations will not effectively enter the cell. Thus, it was necessary to use higher concentrations of catecholamine to observe intracellular transport in the transfected CHO cells. If radiolabeled MPP+ is diluted with unlabeled MPP+ to obtain the concentrations required for significant low-affinity uptake, the amount of radioisotope entering the cell is too low to measure reliably.

We have devised a method to measure intracellular compartmentalization of exogenous catecholamines. CHO cells are exposed to high concentrations of dopamine (1 mM) for 24 hr on glass coverslips, washed, and examined for glyoxylic acid-induced histofluorescence (27, 28). The high concentration of dopamine used to load the cells enters through

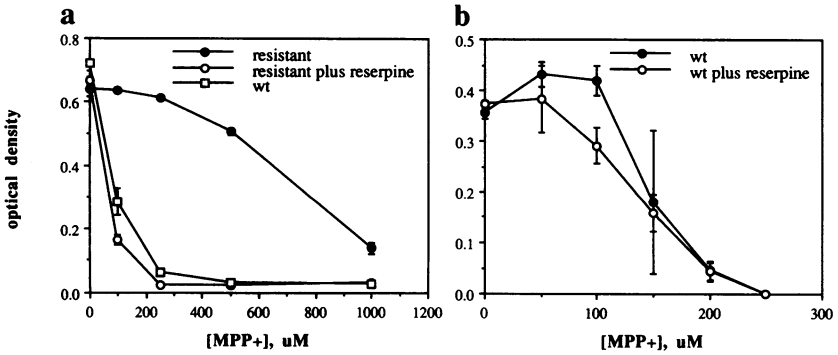

FIG. 3. Reserpine abolishes MPP+ resistance of the CHO transfectant. (*a*) CHO cells at 25% confluence were treated with various concentrations of MPP+ for 3 days in the absence or presence of 1 μM reserpine, and toxicity was assayed as described in Fig. 1. (*b*) Wild-type (wt) CHO cells treated with low concentrations of MPP+ to detect a possible reserpine-sensitive component in their sensitivity.

nonspecific low-affinity systems and thus bypasses the need for high-affinity uptake. Microscopic examination of the loaded cells by the induced fluorescence technique then permits direct visualization of the cellular distribution of exogenous dopamine. When this method is used, wild-type CHO cells show a diffuse, ground-glass pattern of cytoplasmic catecholamine fluorescence (Fig. 4*a*). In contrast, the resistant cells show a very different pattern, with intense accumulation of catecholamine in a perinuclear location (Fig. 4*b*). The cytoplasm also contains a scattered punctate fluorescent stain but less general cytoplasmic fluorescence than wild-type cells, presumably reflecting reduced access of MPP^+ to mitochondria and so accounting for the differences in sensitivity. To determine whether the pattern of catecholamine accumulation in the transfectant was inhibited with reserpine, we performed the same histofluorescence assay using reserpine and found reversion to the wild-type pattern of catecholamine accumulation (Fig. 4*c*). The results indicate that the mechanism of MPP^+ resistance in the transfected cells involves reserpine-sensitive sequestration of the drug within an apparently distinct intracellular compartment.

DISCUSSION

In vivo, systemic administration of MPTP results in the death of nigral dopaminergic neurons but spares other cell populations that also use catecholamines as their neurotransmitter, such as adrenal chromaffin cells, postganglionic sympathetic neurons, and a variety of cell groups in the central nervous system. These cells all express plasma membrane catecholamine transport activity and if this were the sole determinant of toxicity, they would all be susceptible. However, these cells clearly show less susceptibility to systemic MPTP administration than dopaminergic neurons in the midbrain. The differential susceptibility may reflect differences in conversion to MPP^+ by monoamine oxidase. Alternatively, plasma membrane transporters with different activities for MPP^+ may be responsible for the selective vulnerability, and distinct transport proteins for norepinephrine and dopamine have been identified (31–33). However, chromaffin cells, which are relatively insensitive to the toxin, nevertheless demonstrate substantial high-affinity uptake of MPP^+ (17), suggesting that the selective vulnerability cannot be explained solely on the basis of distinct uptake systems. Mechanisms reducing neural activity (15) or compensating for the inhibition of respiration may also play a role in differential cell susceptibility (29), but their physiological significance remains uncertain.

We have used gene transfer to identify a mechanism capable of conferring substantial resistance to MPP^+. This approach makes relatively few assumptions and has the potential to indicate the more functionally significant mechanisms. The reversal of MPP^+ resistance by reserpine and the intracellular accumulation of loaded dopamine suggest that resistance to the toxin derives from expression of a vesicular amine transporter. This transport activity presumably reduces the cytoplasmic level of toxin by sequestering MPP^+ inside an intracellular compartment, thus decreasing the amount of drug available to enter mitochondria and inhibit respiration. Although previous studies have shown that MPP^+ and drugs that inhibit vesicular amine uptake have additive effects on amine depletion (17), the demonstration that this transport activity suffices to protect the cell from MPP^+ toxicity implicates the vesicular amine transporter as a major determinant of resistance among aminergic cell populations. Furthermore, this activity confers resistance to MPP^+ even in a nonneuronal cell line that lacks the synaptic vesicles in which such a transporter normally functions.

Since CHO cells lack synaptic vesicles, the expression of a vesicular transport protein in this fibroblast line would be expected to occur within an anomalous part of the secretory pathway. The transporter acts as a proton exchanger, so functional expression presumably occurs only in acidic compartments, such as the lysosomes, late endosomes, and Golgi complex (34). The perinuclear and punctate cytoplasmic pattern of dopamine-loaded fluorescence supports these locations for expression of the vesicular transporter in the CHO fibroblasts. The low frequency and late appearance of resistant cells during the selection procedure may reflect this heterologous expression as well. Of all the stable transformants containing the same putative cDNA, it seems likely that only a few would express levels high enough to function effectively in a nonneuronal cell type. Alternatively, the development of resistance to MPP^+ may require adaptation by the cell to support sufficient vesicular transport activity. However, the failure to isolate resistant clones by selection of untransfected CHO cells or selection of cells transfected with plasmid vector alone suggests that the mechanism of resistance derives from PC12 cell sequences. In addition, PC12 cells normally express a reserpine-sensitive vesicular amine transporter (18).

The results implicate the balance between plasma membrane and vesicular uptake of catecholamines as a crucial determinant of MPP^+ toxicity. Although a number of aminergic populations accumulate the toxin by high-affinity plasma membrane uptake, it is possible that only nigral neurons degenerate because they express lower levels of vesicular uptake. Thus, although we have identified a component of resistance to the toxin using expression in a fibroblast, its reduced activity in midbrain neurons relative to the adrenal gland (and perhaps sympathetic ganglia) may account for the selective vulnerability of nigral cells. Indeed, chromaffin granule amine content and uptake activity vastly exceed that observed in synaptic vesicles from the central

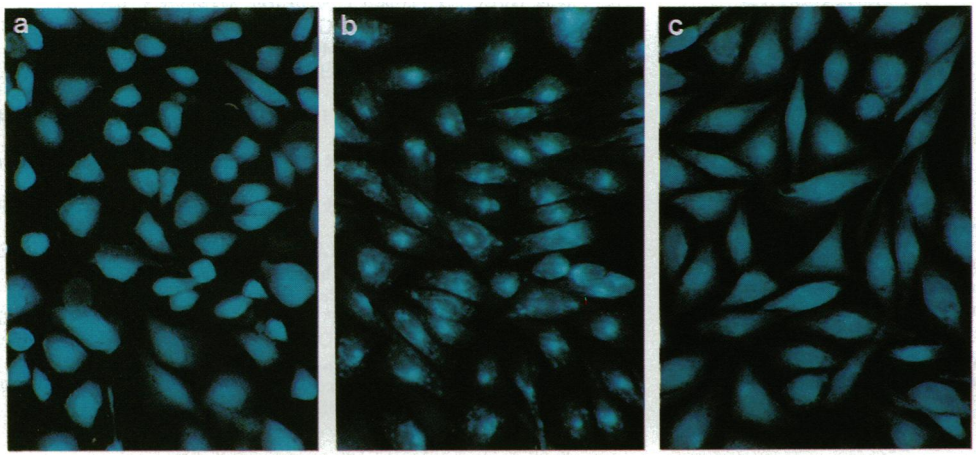

FIG. 4. The MPP^+-resistant CHO transformant shows particulate staining of exogenous dopamine. Wild-type (*a*) and MPP^+-resistant (*b* and *c*) CHO cells were incubated in 1 mM dopamine for 24 hr and the accumulated catecholamine was visualized by glyoxylic acid-induced fluorescence. Wild-type cells show diffuse cytoplasmic staining, and MPP^+-resistant cells show strong perinuclear and particulate cytoplasmic staining, with reduced background cytoplasmic fluorescence. This localized pattern reverts to wild type in the presence of 1 μM reserpine (*c*).

nervous system, suggesting that such differential expression exists and may well account for the observed differential susceptibility to MPP^+.

PD usually spares chromaffin cells of the adrenal medulla and postganglionic sympathetic neurons, so differential expression of the vesicular amine transporter may have relevance for this idiopathic disorder as well. Since it has been proposed that dopamine itself induces oxidative stress (14), an imbalance between membrane and vesicular amine transport activities leading to high cytoplasmic levels of dopamine could be responsible for oxidative stress unrelated to an exogenous toxin (Fig. 5). If this were true, the current work may indicate that manipulation of the vesicular amine transporter has therapeutic potential, independent of the precise toxin involved. The vesicular transporter presumably recognizes the same features of an exogenous or endogenous toxin that are recognized by the plasma membrane transporter. However, in contrast to the plasma membrane transporter, increased expression of the vesicular transporter would have a protective effect.

The availability of transfected cells expressing the vesicular transport system should now make it possible to isolate the sequences responsible for this activity. [^{3}H]Reserpine labels an 80-kDa protein in chromaffin granules and a vesicular transporter for amines has been partially purified and reconstituted in lipid vesicles (35, 36). However, little is known about the molecular basis for this transport activity. Furthermore, neuronal populations expressing other neurotransmitters have distinct vesicular uptake systems (37). These presumably related uptake activities have not been studied nearly as extensively as that for amines. Thus, although classical neurotransmitters require transport into synaptic vesicles for their regulated release in response to neural activity, little is known about this essential neural property.

This work demonstrates that the vesicular transport of a neurotransmitter can be expressed in a cell without synaptic vesicles. Vesicular transport activity apparently does not require other specific synaptic vesicle components for functional expression. It presumably requires only the activity of a more widely distributed vesicular ATPase to generate the proton gradient that drives transport. In addition, the method of loading transfected nonneuronal cells with exogenous neurotransmitter followed by direct visualization of its cellular location provides a method to detect this intracellular transport activity in the absence of a high-affinity plasma membrane transporter. Furthermore, the MPP^+-resistant CHO transfectant now serves as a source for the isolation of sequences that confer vesicular amine transport activity.

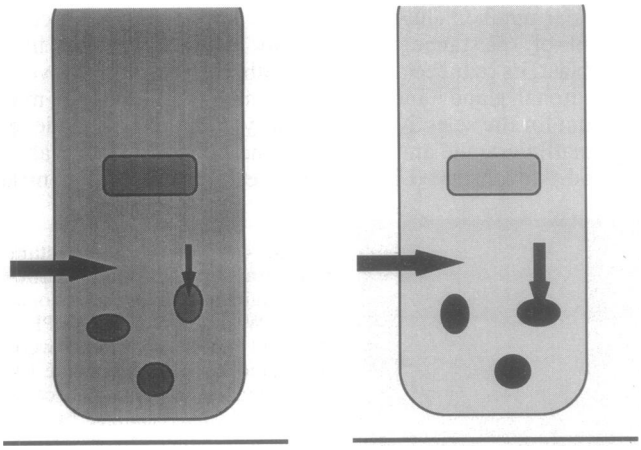

FIG. 5. Balance between plasma membrane and vesicular uptake determines cytoplasmic concentration of MPP^+, its access to mitochondria, and hence toxicity. Diagram shows a presynaptic neuron with mitochondrion, synaptic vesicles, equal plasma membrane uptake, but less (*Left*) and more (*Right*) vesicular uptake. Concentration of toxin or neurotransmitter in the various compartments is indicated by intensity of shading.

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