TOPA Quinone, a Kainate-like Agonist and Excitotoxin, Is Generated by a Catecholaminergic Cell Line

Timothy A. Newcomer,[1] Paul A. Rosenberg,[2] and Elias Aizenman[1]

[1]Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261 and [2]Department of Neurology, Children's Hospital and Harvard Medical School, Boston, Massachusetts 02115

The quinone derivative of 2,4,5-trihydroxyphenylalanine (TOPA) is a selective non-NMDA agonist and excitotoxin. While 3,4-dihydroxyphenylalanine (DOPA)-containing physiological solutions have been shown to generate TOPA and TOPA quinone (TOPA compounds), there have been no previous reports demonstrating the formation of this toxin in biological preparations. Here, using a pheochromocytoma catecholaminergic clonal cell line (PC12), we have identified TOPA compounds as by-products of catecholamine synthesis. PC12 cells incubated for 45 min with 30 μM tyrosine as a catecholamine precursor produced 1.0 ± 0.2 pmol/10⁶ cells of total TOPA compounds. The formation of these compounds could be enhanced nearly twofold when the cells were stimulated with 56 mM KCl. Moreover, the addition of a DOPA decarboxylase inhibitor (30 μM NSD-1015) increased the formation of TOPA compounds in both the unstimulated and stimulated conditions to a maximum of 5.5 ± 0.7 pmol/10⁶ cells after a 45 min incubation. A time-course analysis revealed that DOPA production above baseline levels coincided with the detectable generation of TOPA compounds. Finally, we observed an inhibition of TOPA compounds formation by 100 μM reduced glutathione, suggesting that these catecholamine products are formed from the extracellular autoxidation of DOPA. We propose that TOPA quinone may be an underestimated component of catecholamine toxicity that could be partly responsible for the demise of neurons in several neurodegenerative disorders, including Parkinson's and Huntington's disease. In addition, TOPA quinone may represent the first identified selective non-NMDA agonist that may be synthesized in the brain.

[Key words: DOPA, TOPA quinone, non-NMDA, glutamatergic, excitotoxicity, catecholamine toxicity, PC12, HPLC]

Neuromelanin deposition within the cytoplasm of catecholamine-synthesizing neurons is indicative of nonenzymatic oxidative degradation of these substances (Graham, 1979). The oxidative processes present in these neurons are also likely to be responsible for the generation of reactive oxygen species as well as quinone and semiquinone catechol derivatives. These toxic substances may contribute to the pathogenesis of disorders affecting catecholaminergic neurons or their targets (Cohen et al., 1974; Graham, 1984; Youdim et al., 1993). Our laboratories have been investigating the toxic properties of the quinone derivative of 2,4,5-trihydroxyphenylalanine (TOPA), a proposed intermediate of 3,4-dihydroxyphenylalanine (DOPA) oxidation (Graham and Jeffs, 1977). We have demonstrated that at physiological pH, TOPA rapidly oxidizes to TOPA quinone, a compound that is stable for hours in aqueous solutions (Rosenberg et al., 1991; Kano et al., 1993; Newcomer et al., 1993). TOPA quinone is a selective non-NMDA glutamatergic agonist that can elicit kainate-like electrical responses in neurons (Biscoe et al., 1976; Aizenman et al., 1990; Olney et al., 1990), as well as effectively displace [^{3}H]-AMPA binding in rat striatum (Cha et al., 1991). In addition, excessive stimulation of non-NMDA receptors by TOPA quinone has been shown to be neurotoxic *in vitro* (Olney, 1990; Rosenberg et al., 1991; Aizenman et al., 1992; Skaper et al., 1993a,b).

In a recent study, we observed that DOPA in physiological salt solutions can readily autoxidize to form TOPA and TOPA quinone (Newcomer et al., 1993). This conversion was enhanced 10-fold by strong oxidizing conditions (Newcomer et al., 1995). Although these findings strongly suggest that TOPA quinone may be generated endogenously by DOPA-containing neurons, heretofore, no studies have shown this to be the case. In the present investigations, we have utilized a model catecholaminergic cell system, the PC12 cell line derived from a rat pheochromocytoma (Greene and Tischler, 1976), in order to begin to carefully evaluate the conditions necessary to produce TOPA quinone *in vivo*. The detection of TOPA quinone produced by this system would suggest that this metabolite could be important in a neurodegenerative cascade involving catecholaminergic nuclei or their targets.

Materials and Methods

Materials. TOPA, L-DOPA, L-tyrosine, *m*-hydroxybenzylhydrazine·2HCl (NSD-1015), and reduced glutathione were obtained from Sigma. Mobile phase reagents were HPLC grade, if offered, and obtained from Fisher Scientific, except for sodium 1-octanesulfonic acid (SOS; Eastman Kodak Company) and HCl (Ultrapure, J. T. Baker). Standard stock solutions of 1 mM TOPA and 10 mM DOPA were prepared in 0.01 M HCl, passed through a 0.22 μm filter, and divided into 1.5 ml microcentrifuge tubes for storage at −80°C. Single aliquots were thawed and used for generating standard curves for one day's experiments.

Cell culture. PC12 cells were grown on rat tail collagen-coated 100 mm plastic tissue culture dishes in a growth medium consisting of 85%

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Correspondence should be addressed to Dr. Elias Aizenman, Department of Neurobiology, E1456 BST, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261.

RPMI 1640 (Sigma), 10% heat-inactivated horse serum (Sigma), 5% fetal calf serum (Sigma), 50 μg/ml streptomycin, and 50 U/ml penicillin. Cultures were maintained at 37°C in an atmosphere of 95% O_2/5% CO_2. Culture medium was replaced three times weekly. For experiments, cells were split and plated on collagen-coated 35 mm tissue culture dishes in the same growth medium. Two days after plating the medium was replaced and experiments were performed 24 hr later. During the course of our studies we noticed significant variability in our results if different plating densities were utilized. Similarly, many other groups have also reported that different plating densities of PC12 cells leads to variability in a number of assays (Lucas et al., 1979; Biocca et al., 1980; Tischler et al., 1983; Peppers and Holz, 1986; Vetter and Betz, 1989; Kozak and Yavin, 1992). Therefore, all results presented here were obtained from cultures plated at densities that resulted in 0.5–1 × 10^6 cells/dish the day of the experiment. Platings that resulted in densities outside this range were not used.

Measurement of production of TOPA compounds. Cells were washed twice with 1 ml of Hanks' balanced salt solution (HBSS; 137 mM NaCl, 4 mM $NaHCO_3$, 0.34 mM Na_2HPO_4, 5.37 mM KCl, 0.44 mM KH_2PO_4, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.60 mM glucose, 25 mM HEPES, adjusted to pH 7.4 with NaOH), and incubated with 0.9 ml of an exposure medium consisting of 30 μM tyrosine in regular or high K^+ HBSS, in the presence or absence of drugs. For the high K^+ (56 mM) exposure medium, there was a corresponding decrease in NaCl to maintain a constant osmolarity. The cultures were placed into the 37°C incubator for the appropriate time of exposure. Experiments were terminated by placing the culture dishes on ice and adding 0.1 ml of 2 N HCl to each dish. The cells were then scraped from the dishes and the suspensions transferred to ice-chilled 1.5 microcentrifuge tubes. The tubes were sonicated for 10 sec to ensure cellular disruption and spun at 12,000 × *g* for 5 min at 4°C. Two hundred microliters of the supernatant were passed through 5000 nominal molecular weight cutoff filter microcentrifuge tubes at 12,000 × *g* for 15 min at 4°C and assayed for the presence of catecholamines. For all experiments, cell counts were performed on sister cultures that were treated similarly to the experimental cultures and detached with 0.25% trypsin.

Catecholamine analysis. HPLC procedures were similar to those previously described (Newcomer et al., 1993). Briefly, separation was achieved using a reverse-phase guard column and an analytical column (4.6 mm i.d. × 25 cm long, Rainin) packed with C18 modified silica particles 5 μm in diameter. Samples (25 μl) of solutions were introduced into the column using a manual injector. The mobile phase comprised a mixture of 100 mM phosphoric acid, 0.1 mM Na_2EDTA, 2.0 mM SOS (with the pH adjusted from 2.4 to 3 with NaOH) and 12–15% (v/v) methanol. Compounds were measured with an electrochemical detector (Coulochem II, ESA) composed of dual coulometric porous graphite electrodes (model 5014, ESA) that followed a conditioning cell (model 5021, ESA). Electrode settings were adjusted to screen for TOPA compounds as follows: conditioning electrode = −100 mV, electrode 1 = −100 mV, and electrode 2 = −350 mV (Newcomer et al., 1993). Peak identity was established utilizing the following criteria: (1) retention time at various chromatography conditions as indicated in the results, (2) addition of authentic standard to increase peak size (spiking), and (3) electrochemical behavior of standard versus unknown at different electrode potentials. Quantification was performed by integrating the area under a peak relative to the baseline, and then calibrating with standard curves. The limit of sensitivity, defined as a signal-to-noise ratio > 2, was 2.5 fmol for TOPA and TOPA quinone. DOPA was similarly measured as above, except using the following electrode settings: conditioning cell = +150 mV, electrode 1 = +150 mV, and −350 mV.

Results

Our initial set of experiments were designed to investigate whether PC12 cells could produce TOPA and TOPA quinone, and whether these substances could be identified reliably by an HPLC assay (Newcomer et al., 1993). Working on the premise that a large amount of DOPA production would coincide with significant formation of TOPA compounds, PC12 cells were stimulated under conditions previously shown to be effective in generating DOPA (Lee et al., 1985). Cultures were thus exposed to an incubating medium consisting of the catecholamine precursor tyrosine (30 μM) and the aromatic amino acid decarboxylase inhibitor NSD-1015 (30 μM) in 56 mM K^+ HBSS. After a 45 min exposure, the incubation was terminated and the samples prepared for HPLC analysis. We began with chromatography conditions (Newcomer et al., 1993) that previously allowed us to separate TOPA and TOPA quinone from other catecholamine-related substances such as norepinephrine (NE), DOPA, epinephrine (EPI), 3,4-dihydroxyphenylacetic acid (DOPAC), methyl-tyrosine (M-TYR), dopamine (DA), and homovanillic acid (HVA). These conditions consisted of utilizing a mobile phase buffered to pH 3 and containing 15% methanol (mobile phase A) and setting the electrodes at the detector at potentials (electrode 1 = −100 mV and electrode 2 = −350 mV) that substantially diminish the signals produced by NE, DOPA, EPI, DOPAC, M-TYR, DA, and HVA, but not by TOPA or TOPA quinone (Newcomer et al., 1993). Under these circumstances (Fig. 1, left), retention times for TOPA quinone and TOPA standards were, respectively, 6.1 and 7.4 min. In addition, the typical dominant species of authentic TOPA solutions prepared at physiological pH is TOPA quinone (Newcomer et al., 1993). In the samples from the stimulated PC12 cells there were also two peaks with corresponding retention times. When the PC12 samples were mixed with equal parts of the standard (spiking), the corresponding peaks remain elevated while the other peaks in the samples were diminished.

To confirm the positive identification of TOPA quinone and TOPA, chromatography conditions were altered to further separate the peaks of interest from all other confounding peaks (Fig. 1, right). Specifically, the pH of the mobile phase was lowered to 2.4 and the methanol concentration was decreased to 12% (mobile phase B). This resulted in an increased retention time for both TOPA and TOPA quinone (10.0 and 11.1 min, respectively, Fig. 1, right). Using these conditions, the appearance and retention times of peaks derived from the unknown samples were identical to a standard of TOPA compounds that had been acidified in a similar fashion to the PC12 cell extract. This procedure had been previously shown to reduce a portion of TOPA quinone back to TOPA (Newcomer et al., 1993). These HPLC separation conditions were utilized for the analysis and quantification of TOPA compounds in PC12 cell samples throughout the remainder of this study. TOPA and TOPA-quinone always coexist as a tautomeric mixture in solution, with TOPA being the dominant species under acidic conditions, while the quinone predominates at physiological or alkaline pH levels (Newcomer et al., 1993). In addition, both these substances can be readily interconverted (Newcomer et. al., 1993). Therefore, in all subsequent quantification of our data, we have pooled the total amount of TOPA compounds detected in the experimental samples.

The next set of experiments were designed to define in more detail the various conditions for the production of TOPA compounds by PC12 cells (Fig. 2). Cultures incubated for 45 min in the presence of the precursor tyrosine (30 μM) alone in HBSS generated a small but measurable amount of total TOPA compounds (1.0 ± 0.2 pmol/10^6 cells; mean ± SEM, n = 4). Since it seemed reasonable to assume that TOPA compounds were directly derived from DOPA oxidation (Graham and Jeffs, 1977; Newcomer et al., 1993), incubation conditions were altered to either increase DOPA synthesis or to allow for its accumulation. The DOPA-synthesizing enzyme tyrosine hydroxylase is activated by phosphorylation via several Ca^{2+}-dependent protein kinases (Funakoshi et al., 1991). Therefore, when PC12 cells are depolarized with high K^+ solutions, the ensuing Ca^{2+} entry via

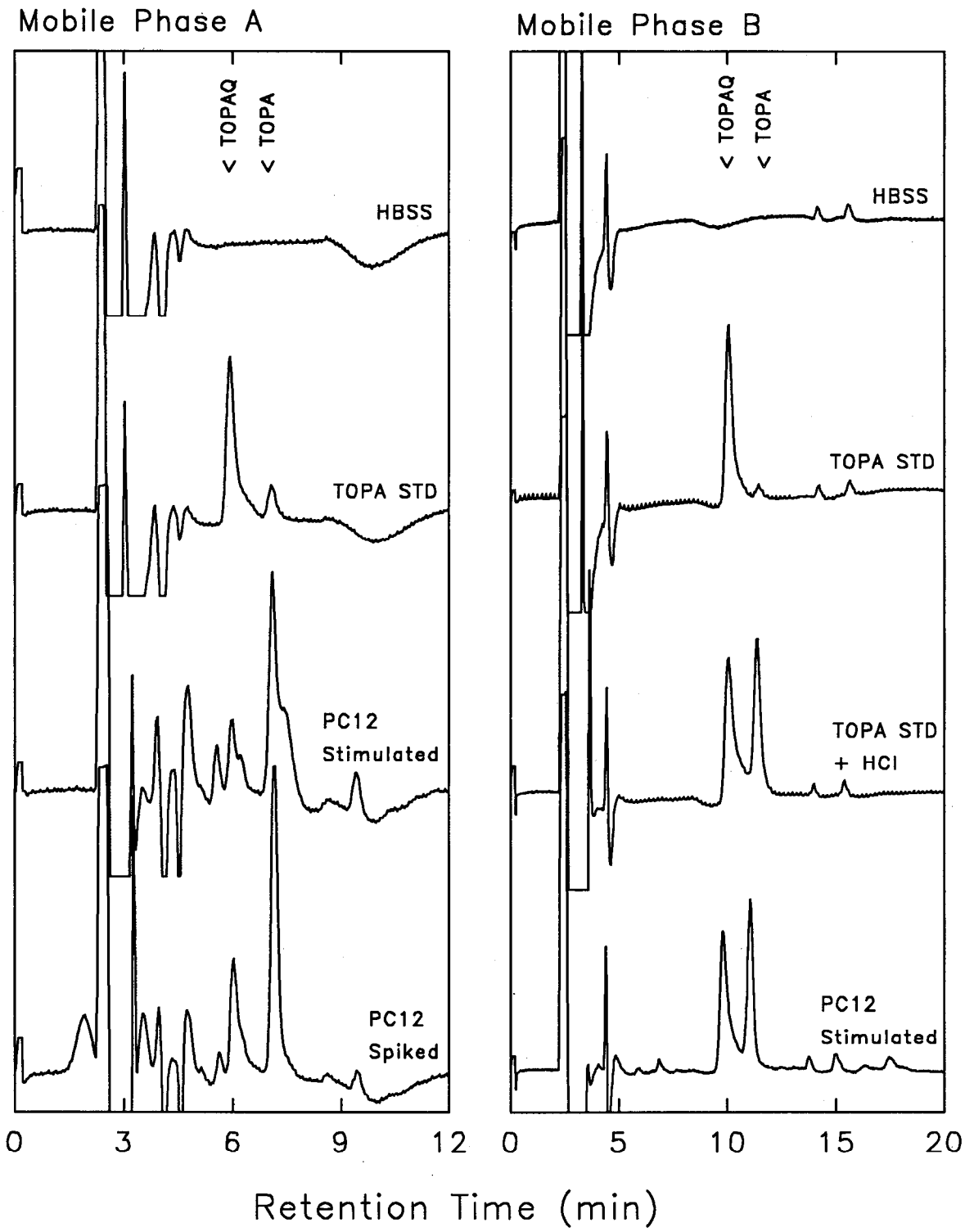

Figure 1. Identification of TOPA and TOPA quinone in PC12 cells. Shown on the *left* are four chromatograms obtained using *mobile phase A* (pH 3 and 15% methanol). *HBSS*, a chromatogram of an incubation medium blank containing 30 μM tyrosine and 30 μM NSD in a high K^+-HBSS. *TOPA STD*, TOPA diluted to 30 nM (0.75 nmol) in the HBSS incubation medium at physiological pH showing the typical dominant species as TOPA quinone; retention times for TOPA quinone and TOPA are 6.1 and 7.4 min, respectively. *PC12 Stimulated*, extracts from PC12 cells exposed to the above incubation medium for 45 min and then exposed to HCl have two corresponding peaks with comparable retention times to the standard. *PC12 Spiked*, a 50:50 mixture of the TOPA standard, and the PC12 cells sample kept the corresponding peaks in the sample elevated without broadening them while all other peaks were diminished. The *right panel* displays four chromatograms obtained using *mobile phase B* (pH 2.4, 12% methanol). *HBSS, TOPA STD, and PC12 Stimulated*, chromatograms obtained from samples similar to those described above. *TOPA STD + HCl*, a 50 nM TOPA standard was acidified in a similar matter as the PC12 samples, demonstrating how a portion of TOPA quinone is converted back to TOPA; the standard now parallels the appearance and retention times of the PC12 cell sample.

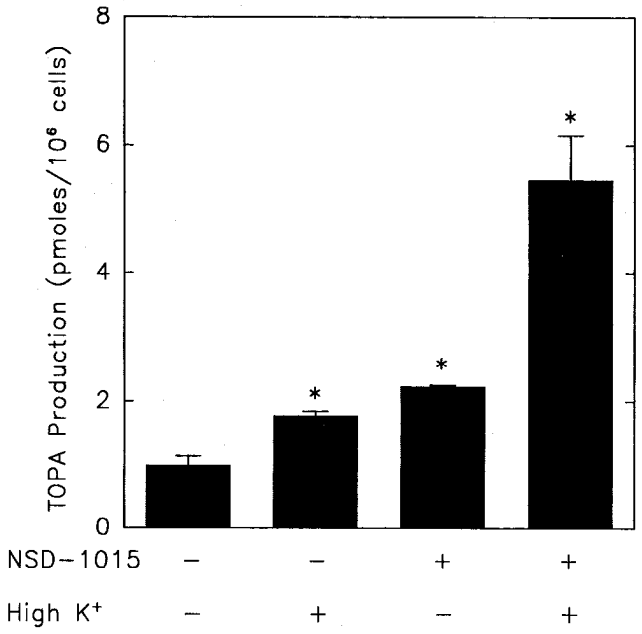

Figure 2. Conditions for formation of TOPA compounds in PC12 cells. PC12 cells were incubated for 45 min in a HBSS or high K^+-HBSS containing 30 μM tyrosine in the presence and absence of 30 μM NSD-1015. Total TOPA compounds measured are expressed as mean ± SEM of three to six experiments. The *asterisk* (*) indicates a one-way analysis of variance revealed a significant difference among groups ($p < 0.001$; $F = 13.6$). Post hoc comparisons among selected groups revealed significant differences ($p < 0.005$) between treatment groups and control.

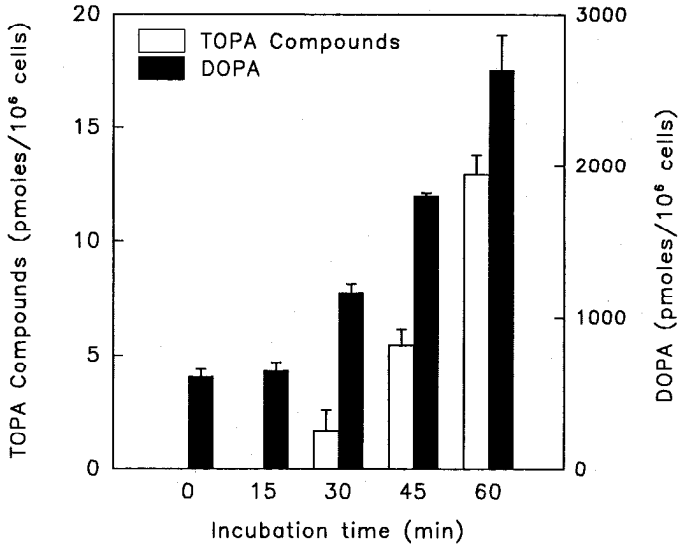

Figure 3. Time course of formation of DOPA and TOPA compounds. PC12 cells were incubated in a medium containing 30 μM tyrosine and 30 μM NSD-1015 in high K^+-HBSS. At the indicated time points, the incubation was stopped and the resultant total DOPA and TOPA compounds measured. Shown are mean ± SEM of three to six experiments per time point.

voltage-gated channels results in a rapid, increased activation of tyrosine hydroxylase (Yanagihara et al., 1984; Nose et al., 1985). Under these conditions, TOPA compounds production was increased nearly twofold in these cells. Moreover, this production was similarly increased by utilizing a DOPA decarboxylase inhibitor (30 μM NSD-1015), and, in fact, the combination of both a high K^+ stimulation and decarboxylase inhibition resulted in a fivefold increase in TOPA production after a 45 min incubation (Fig. 2).

The temporal relationship between DOPA synthesis and production of TOPA compounds was next examined. PC12 cells were exposed to an incubating medium to optimally produce DOPA, containing 30 μM tyrosine, 30 μM NSD-1015, and high K^+. The incubations were terminated at 0, 15, 30, 45, and 60 min, and the PC12 samples analyzed for total DOPA and TOPA compounds (Fig. 3). Some DOPA was detectable at time 0, which reveals the basal levels of this substance that these cells can produce. The production of DOPA did not significantly increase over baseline until 30 min of incubation, at which time TOPA compounds could be detected for the first time. The production of DOPA and TOPA compounds continued to increase with time. After 1 hr, 12.9 ± 0.9 pmol/10^6 cells ($n = 6$) of TOPA compounds could be measured, which represents approximately 1% of the amount of DOPA compounds that were produced.

It has been reported that the majority of DOPA is found extracellularly during incubation conditions similar to those described above (Greene and Rein, 1977). In addition, the aforementioned lag in the detection of TOPA compounds that coincides with DOPA production above baseline levels suggests that TOPA compounds may be formed from the autoxidation of extracellular DOPA. This possibility was tested by utilizing reduced GSH in the incubation media, as this antioxidant does not permeate cell membranes and is not significantly transported into most cells (Meister, 1994). GSH, as well as other reducing agents such as dithiothreitol, cannot only prevent TOPA quinone formation from TOPA (Rosenberg et al., 1991; Aizenman et al., 1992), but can also inhibit TOPA production from DOPA in physiological salts solutions (Newcomer and Aizenman, unpublished observation). PC12 cells were incubated for 60 min in a medium containing 30 μM tyrosine and 30 μM NSD-1015 in high K^+ HBSS in the presence and absence of 100 μM GSH. Although this thiol did not alter the accumulation of DOPA in the cultures, it completely blocked the production of TOPA compounds (Fig. 4).

Discussion

These experiments demonstrate, for the first time, that the excitotoxin TOPA quinone, as well as its precursor TOPA, can be formed from a cellular source of DOPA. Even PC12 cells presented only with the substrate tyrosine were capable of producing TOPA compounds as by-products of catecholamine synthesis. This production was increased when the PC12 cells were stimulated with high K^+ HBSS to increase tyrosine hydroxylase activity and thereby increase DOPA synthesis along with its release. Furthermore, when DOPA was allowed to accumulate by preventing its degradation with NSD-1015, there was a further increased formation of TOPA compounds in both unstimulated and stimulated conditions.

It is noteworthy that PC12 cells represent a simple model system, and it will be important to determine whether similar results can be obtained with either cultured mesencephalic neurons or in more intact systems. Nevertheless, evidence from this study strongly suggests that TOPA compounds form following the extracellular autoxidation of DOPA. It is likely that baseline DOPA levels detected are mostly intracellular where formation of TOPA compounds would be expected to be inhibited by in-

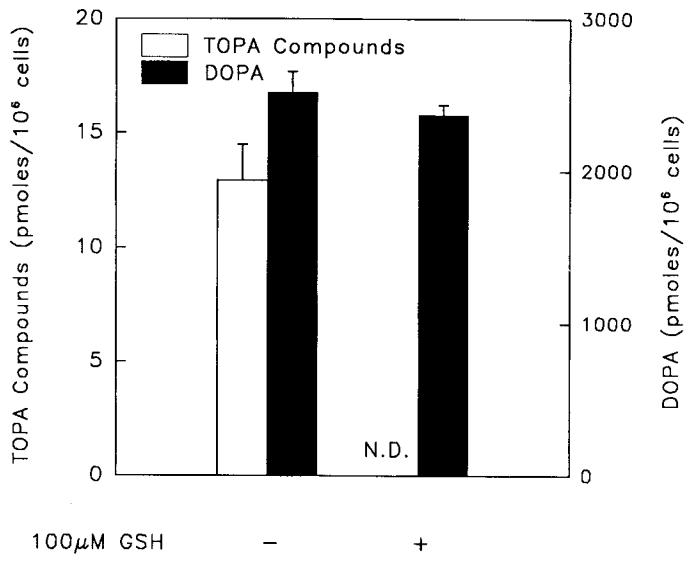

Figure 4. GSH prevents the production of TOPA compounds. PC12 cells were incubated in a medium containing 30 μM tyrosine and 30 μM NSD-1015 in high K^+-HBSS in the presence and absence of 100 μM GSH. After 60 min, the incubation was terminated and the resultant total content of DOPA and TOPA compounds measured. Values are expressed as mean ± SEM of four to six experiments. *N.D.*, not detectable.

tracellular antioxidants. Upon continued stimulation, any DOPA that accumulates extracellularly (Greene and Rein, 1977) can autoxidize to generate TOPA and TOPA quinone, a reaction that can be inhibited by GSH. Therefore, it is readily apparent from our results that TOPA and TOPA quinone are not stored or accumulated inside cells, since extracellular GSH completely abolished their detection. It is possible, nonetheless, that GSH could be reacting with the TOPA compounds after they are formed and produce adducts, thereby appearing as if oxidation had not taken place. In fact, GSH has been reported to react with semiquinone free radicals and ortho-quinones (Mans et al., 1992), although it does not react with catechols (Mans et al., 1992), and, thus, at least some unoxidized TOPA would still be expected to be present in the GSH-containing media under any circumstances.

The demonstration of the formation of TOPA compounds in a catecholaminergic cell line in culture suggests that these substances could be formed in the brain. It is noteworthy that DOPA itself has been shown to be released from nicotine-stimulated striatal slices obtained from rats (Misu et al., 1990) and can be detected after K^+-evoked release in rat striatum by microdialysis (Nakamura et al., 1992). It is reasonable to think that TOPA compounds could be formed from these extracellular DOPA sources in a similar manner to that reported in this study, especially in an oxidative environment (Newcomer et al., 1995). Such redox conditions could exist in some pathological states and, in fact, have been associated with the etiology of disorders such as Parkinson's disease (Jenner et al, 1992; Olanow, 1992). Our aforementioned results with GSH are also interesting in light of its known association with cellular protection systems (Meister, 1994). For example, a glutathione deficiency has been proposed to be associated with mitochondrial damage or poisoning, leading to oxidative stress (Jain et al., 1991; Mithöfer et al., 1992). Moreover, a nigral glutathione deficit has been implicated in the pathogenesis of Parkinson's disease (Perry et al., 1982; Sofic et al., 1992). GSH has further been shown to be released from neurons in brain slices from rats (Zängerle et al., 1992), suggesting an extracellular antioxidant role for this substance. Hence, any DOPA present intracellularly or extracellularly during conditions when tissue antioxidant defenses are compromised may lead to the formation of TOPA compounds.

It is of special interest that there have been no previous reports of a brain-derived agonist specific for non-NMDA glutamatergic receptors. However, there is evidence that points to the specific involvement of non-NMDA receptors in playing a role in certain neurodegenerative disorders such as global cerebral ischemia (Sheardown et al., 1990), motor neuron disease (Couratier et al., 1993; Shaw et al., 1994), neurolathyrism (Olney et al., 1976; Spencer et al., 1986; Bridges et al., 1989), and mussel poisoning (Perl et al., 1990; Teitelbaum et al., 1990). Furthermore, it has been clearly demonstrated that activation of non-NMDA receptors in cultured neurons and brain slices can lead to excitotoxic cell death (Koh et al., 1990; Garthwaite and Garthwaite, 1991a,b). Of interest, it has been observed that a prior lesion of the substantia nigra protects striatal neurons from transient ischemia (Globus et al., 1987), potentially linking a catecholaminergic system with a lesion that is normally associated with glutamate toxicity (Rothman and Olney, 1986). It is thus possible that the generation of TOPA quinone by dopaminergic cells could render neighboring somata or afferent projection neurons vulnerable to excitotoxic injury via selective activation of non-NMDA receptors.

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