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19. In release experiments potassium or veratrine also failed to release several other nontransmitter amino acids including taurine, threonine, serine, valine, leucine, isoleucine, tyrosine, phenylalanine, histidine, lysine, and arginine.
20. Concentrations of many other amino acids including taurine, threonine, serine, valine, methionine, leucine, isoleucine, tyrosine, phenylalanine, histidine, lysine, and arginine were also measured and found to be identical in unaffected and myoclonus spinal tissue (NS by *t* test).
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24. Spinal cord membranes were prepared essentially as described (*9*). Frozen tissue was thawed, homogenized in 0.32*M* sucrose, and centrifuged at 27,000*g* for 20 min. The pellet was then washed three times by resuspension in distilled water and resuspended at 20 mg (original wet weight) per milliliter in 50 m*M* sodium potassium phosphate buffer, *p*H 7.4, containing 200 m*M* NaCl, for use in binding assays. [^{3}H]Strychnine (30.0 Ci/mmol, Amersham; 1 to 35 n*M* or 4 n*M* plus 1 to 1000 n*M* unlabeled strychnine) was incubated with membranes in a final volume of 0.5 ml for 15 min at 4°C. Nonspecific binding was measured in the presence of 10 m*M* glycine. Incubations were terminated by filtration onto glass fiber filters (Schleicher Schuell, no. 32), and samples were rapidly washed three times with 3-ml aliquots of ice-cold 0.15*M* NaCl. Filter-bound radioactivity was measured by scintillation spectrometry. Data were analyzed with the computer programs EBDA and LIGAND [G. A. McPherson, *Comput. Programs Biomed.* **67**, 107 (1983)] to obtain values for K_d and B_{max}.
25. For [^{3}H]strychnine autoradiography, sections (10 μm) were incubated for 30 min at room temperature in 50 m*M* sodium potassium phosphate buffer, *p*H 7.4, containing 200 m*M* NaCl, and then with 3 n*M* [^{3}H]strychnine in the same buffer, in the absence and presence of 10 m*M* glycine for 25 min at 4°C. Sections were washed for 4 min in ice-cold buffer and dried [M. A. Zarbin, J. K. Wamsley, M. J. Kuhar, *J. Neurosci.* **1**, 532 (1981)]. For [^{3}H]flunitrazepam, sections were processed as described [J. R. Unnerstall, M. J. Kuhar, D. L. Niehoff, J. M. Palacios, *J. Pharmacol. Exp. Ther.* **218**, 797 (1981); (*16*)]. Autoradiograms were produced by apposing labeled sections to Ultrofilm (LKB) for 8 to 12 weeks for both [^{3}H]strychnine and [^{3}H]flunitrazepam.
26. Synaptosomes were purified from 10% homogenates of rapidly thawed tissue in 0.32*M* sucrose as described (*10*) and resuspended in ice-cold 0.32*M* glucose. In each preparation, the sodium-dependent, high-affinity uptake of both [^{3}H]glycine (19.0 Ci/mmol, Amersham) and [^{3}H]glutamate (53.0 Ci/mmol, Amersham) were measured by incubating synaptosome suspensions in Krebs-Hepes buffer for 2 min at 37°C; control incubations were carried out in medium in which all sodium ions were replaced by tris (*18*).
27. For release studies, synaptosomes (*10*) were incubated at 37°C for 20 min in Krebs-bicarbonate buffer containing 2.5 m*M* Ca^{2+}. Portions of KCl solution (to give a final concentration of 47.5 m*M*) or veratrine solution (to give a final concentration of 75 μ*M*) were then added, and the incubation continued for an additional 10 min. Addition of NaCl solution or normal medium served as the respective controls. Amino acid levels were determined in acidified samples of medium, after brief centrifugation at room temperature, on a Varian high-performance liquid chromatograph by means of cation exchange in lithium buffers and post-column derivatization with *ortho*-phthalaldehyde.
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Synaptic Transmission Between Dissociated Adult Mammalian Neurons and Attached Synaptic Boutons

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In most studies of synaptic currents in mammalian central neurons, preparations have been used in which synaptic currents are recorded at some distance from the synapse itself. This procedure introduces problems in interpretation of the kinetics and voltage-dependent properties of the synaptic current. These problems have now been overcome by the development of a preparation in which presynaptic vesicle-containing boutons have been coisolated with the soma of individual neurons, thus providing the opportunity to study synaptic currents under conditions of both adequate voltage control and internal ionic perfusion. Spontaneous synaptic currents mediated by γ-aminobutyric acid and excitatory amino acids were recorded from neurons isolated from a mammalian medial solitary tract nucleus. Calcium- and depolarization-dependent spontaneous currents of several to hundreds of picoamperes occurred with rapid rise times of 0.8 to 3 milliseconds and decays at least ten times as long.

THE USE OF DISPERSED ADULT MAMmalian neurons to characterize the properties of central neurons is becoming widespread (*1*). Normally, the synaptic inputs can only be determined indirectly through the response to application of exogenous transmitter substances. However, we report here a neuronal isolation procedure that leads to retention of synaptic function.

Neurons isolated from the medial, dorsomedial, and commissural subnuclei of the medial nucleus tractus solitarius (MNTS) had cell bodies 10 to 13 μm in diameter and bipolar processes of 10 to 100 μm (Fig. 1A). A similar morphology is predominant in stained sections from the MNTS (*2*). Structures were apparent that were the synaptic endings of the in vivo afferent inputs to these neurons (Fig. 1B). These boutons have retained vesicles believed to be associated with neurotransmitter release. Retention of vesicles was dependent on isolation and incubation of the tissue in a low Ca^{2+} (0 to 200 μ*M*) and high Mg^{2+} (5 m*M*) solution, rather than solutions containing 1 to 2 m*M* Ca^{2+} as used by others (*1*).

Neurons with neurites shorter than 20 μm were selected for our patch clamp studies. These neurons had capacitive charging currents with single time constants and an average capacitance of 6 to 8 pF. These capacitances are consistent with values expected from an ellipsoid geometry and an assumed specific capacitance of 1 μF/cm^2 (*3*). These conditions ensure adequate temporal and spatial control of postsynaptic membrane potentials for the currents we investigated.

Both spontaneous inward and outward current deflections were observed in these neurons at a holding potential of −40 mV, which in their rapid onset and slow decay resembled synaptic currents (*4, 5*). Previous electrophysiological and histochemical experiments were consistent with the possibility that glutamate, or an analog of glutamate (*6*), and γ-aminobutyric acid (GABA) (*7*) were neurotransmitters in the MNTS.

The inhibitory amino acid, GABA (*8*), and the excitatory amino acids (EAA), glutamate or its structural analogs (*9*), are proposed to be the major transmitters at fast synapses in the mammalian brain. GABA activates a Cl^- conductance at the $GABA_A$ receptor (*8, 10*). Glutamate is an agonist for multiple receptors that activate nonspecific cation conductances (*9, 11*). In our conditions, the Cl^- reversal potential was ~ −90 mV and, if equal permeabilities for Na^+, K^+, and Cs^+ (*12*) are assumed, the cation reversal potential was ~0 mV. At a mem-

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brane potential between these two potentials, −40 mV, Cl^--conducting GABA-mediated inhibitory synaptic currents would be outward and EAA-mediated excitatory currents would be inward. By setting the membrane potential at either 0 or −90 mV we could select for one of the two synaptic currents. At a holding potential of −80 mV, close to the Cl^- equilibrium potential (E_{Cl}), spontaneous inward currents were seen. The rise time of this inward (excitatory) current was 0.8 to 3 ms, and the decay was monoexponential with a time constant (τ) of 10 to 35 ms (Fig. 2A). With another neuron, the potential was held at 0 mV, the equilibrium potential for cations (E_{cation}), and spontaneous outward currents were observed. The rise time of this outward (inhibitory) current was also 0.8 to 3 ms, but the τ was slower, 10 to 100 ms (Fig. 2B). Although not all cells were examined at both potentials, 80% of the neurons examined showed at least one of the two currents.

These spontaneous currents had permeability characteristics of EAA-mediated (Fig. 2A) and GABA-mediated (Fig. 2B) synaptic currents. To further examine these possibilities we tested the effects of antagonists for GABA and glutamate. Application of the EAA antagonist γ-D-glutamylglycine (DGG) (2 m*M*) (*13*) resulted in a reversible reduction in the spontaneous inward currents (Fig. 2A). The GABA antagonist picrotoxin (PTX) (50 μ*M*) (*8*) blocked the spontaneous outward currents (Fig. 2B). In the presence of PTX the spontaneous currents had a null potential of about 0 mV, consistent with a nonselective cation current (Fig. 3, A and C). In the presence of DGG the spontaneous currents were outward at potentials greater than −60 mV. The reversal potential appeared to be near −80 mV (Fig. 3, B and D). The two different reversal potentials for the spontaneous currents show that two different conductances, a nonselective cation conductance and a Cl^- conductance, were activated. The blocking effects of DGG and PTX suggest that spontaneous release of glutamate-like and GABA-like neurotransmitters are responsible. Therefore we examined the possibility that transmitter was being released from presynaptic terminals.

Transmitter release is Ca^{2+}-dependent and is initiated by depolarization of the presynaptic terminal (*14*). We therefore tested the effects of external Ca^{2+} concentration and presynaptic depolarization produced by increasing concentrations of K^+ on the spontaneous currents. The neuron was held at −80 mV, a potential where the voltage-dependent Ca^{2+} channels are closed (*15*). The potential of the attached nerve endings, however, was not clamped, and therefore changes in extracellular Ca^{2+} modified Ca^{2+} influx if the presynaptic membrane potential was sufficiently depolarized to allow the Ca^{2+} channels to open. Increasing Ca^{2+} from nominally free to 2 m*M* (not shown) or to 4 m*M* increased the frequency of spontaneous inward currents, and currents of larger amplitude became more frequent (Fig. 4A). Increasing the external K^+ concentration depolarized the presynaptic terminals but did not affect the potential of the voltage-clamped postsynaptic neuron (*16*). This procedure increased the frequency of the GABA-activated spontaneous outward currents (Fig. 4B), as would be expected if

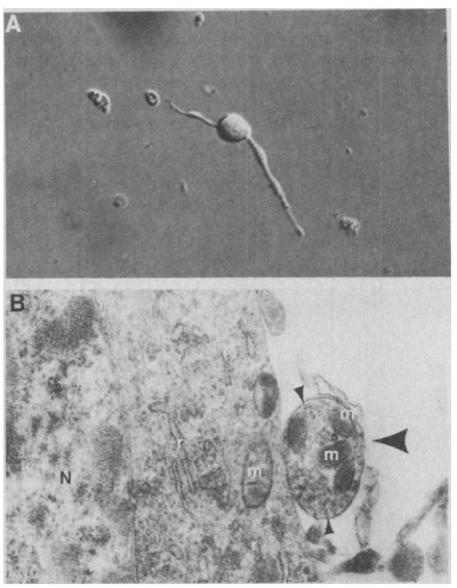

Fig. 1. (**A**) Photograph of a typical adult guinea pig neuron isolated from the MNTS. The isolation procedure is an adaptation of a published technique (*20*). (**B**) Electron micrograph of an isolated MNTS neuron with an attached bouton (large arrowhead) containing numerous synaptic vesicles (small arrowheads), and several mitochondria (m). In the cytoplasm of the cell body are profiles of the nucleus (N), rough endoplasmic reticulum (r), and mitochondria. Small vesicles are seen at or near the attached fiber. The asymmetric synaptic type is similar to that of a baroreceptor afferent (*2*). Isolated neurons were fixed with 4% paraformaldehyde in 0.1 m*M* $NaPO_4$, *p*H 7.4. After washing in phosphate buffer and dehydration in a graded series of ethanols, the neurons were embedded in Araldite 6005 (Electron Microscopy Sciences), and sectioned in semithin or ultrathin sections (×25,000).

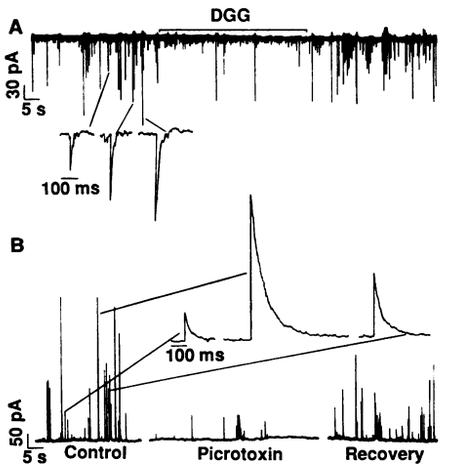

Fig. 2. Spontaneous current fluctuations blocked by an EEA antagonist and a GABA antagonist. (**A**) Reversible block of inward currents by 2 m*M* DGG at a holding potential of −80 mV. (**B**) Reversible block of outward currents by 50 μ*M* PTX at a holding potential of 0 mV. Representative inwardly and outwardly directed spontaneous current deflections are shown on an expanded time scale. Antagonists, diluted in external solution, were applied under positive pressure from a pipette situated within 10 μm of the neuron (*9, 13*). Separation of cation (E_{cation} = 0 mV)- and Cl^- (E_{Cl} = 90 mV)-dependent currents was done with a low Cl^- (4 m*M*) internal solution with methanesulfonate (Eastman Kodak) as the major anion (*10, 11*). The whole-cell patch-clamp technique (*21*) was used for current recordings; the pipette resistance was 3 to 8 megohms. The pipette solution contained: 130 m*M* Cs-$MeSO_3$, 2 m*M* $MgCl_2$, 5 m*M* Na-$MeSO_3$ or Na-ATP, 10 m*M* Cs-Hepes, and 2.5 m*M* EGTA (*p*H adjusted to 7.2 with CsOH). Current and voltage data were filtered at 2 kHz. Results were typical of (A) in three experiments and of (B) in five experiments.

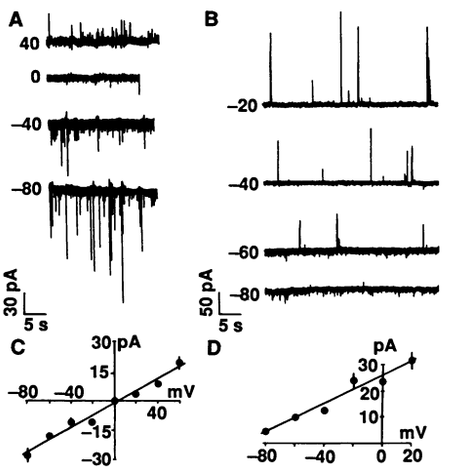

Fig. 3. Reversal potential of spontaneous synaptic events. (**A**) EAA antagonist-sensitive spontaneous synaptic events. PTX (25 μ*M*) was used to block the GABA-activated conductances isolating the EAA-activated (excitatory) currents. The spontaneous synaptic events at the different potentials shown were blocked by DGG (Fig. 2A). (**B**) PTX-sensitive spontaneous synaptic events. DGG (2 m*M*) was used to antagonize the EAA-activated conductances. The neuron was voltage-clamped at the potential indicated, and spontaneous current fluctuations were recorded (*16*). The average amplitude of spontaneous activity for each potential with SEMs are shown. (**C**) Data from (A). (**D**) Data from (B). Lines were fit by linear regression of the mean. Events with an amplitude greater than three times the mean were not included so as not to bias the relation. Results were typical of (A) and (C) in three experiments and (B) and (D) in five experiments.

presynaptic depolarization increases the release of transmitter.

At 0 mV the putative GABA-mediated spontaneous currents had a large dispersion of amplitudes ranging from a minimum of 3 to 4 pA to as large as 500 pA. At this potential, smaller current steps (1.3 pA in amplitude) that were blocked by PTX were also observed. These were interpreted as unitary currents, possibly caused by transmitter leak (*17*), with a slope conductance of 16 pS ($n = 5$) (*18*). This suggests that from 3 to ~400 GABA channels were activated during the spontaneous synchronous release. The τ of the proposed GABA-mediated synaptic currents in other preparations recorded near 0 mV is 20 to 35 ms (*4*), which corresponds to that of the 3 to 5 pA currents that we recorded. We observed an amplitude dependence of the τ for the GABA-mediated events, the larger events having longer τ values (up to 100 ms) (Fig. 2B). The EAA-mediated synaptic currents may vary in a neuron from a few picoamperes to several hundred picoamperes, the average minimum size appearing to be 4 to 6 pA at −80 mV. The τ of the EAA-mediated events recorded at hyperpolarized potentials in other preparations, with the expected temperature dependence taken into account, has ranged from 1 to 12 ms (*5*). The longer τ (10 to 35 ms) could, as for the GABA-mediated currents, result from reactivation of channels because of slower transmitter clearance from the synapse. This slower rate may be due to the lack of cells that are normally involved in reuptake of released transmitter.

The amplitudes of the spontaneous currents appeared to fluctuate in a quantal fashion, and although the frequency distribution occurred in steps, the range of amplitudes made analysis difficult. To limit the range of amplitudes, we studied the spontaneous GABA-mediated currents in nominally zero extracellular Ca^{2+}. The frequency of the currents decreased to an average of 0.1 Hz and their amplitudes decreased to less than 20 pA (Fig. 4C). The miniature GABA-mediated inhibitory postsynaptic currents (mIPSC) were separated into 1-pA bins, and the majority occurred at 3 to 5 pA. The frequency of the mIPSC was then increased by exposure to zero extracellular K^+, which blocks the Na^+ pump and produces presynaptic depolarization (*19*). The frequency was increased up to fourfold, but the amplitude frequency distribution was unchanged (Fig. 4D). These average minimum values are similar to those observed when frequency of spontaneous currents was increased by increased concentrations of Ca^{2+}.

Response of an isolated neuron to an applied transmitter does not confirm its role as a natural transmitter; our method allows this determination. We propose that the DGG-sensitive current fluctuations are caused by a spontaneous release of an EAA transmitter from presynaptic boutons that have remained attached to the neuron during its isolation. We also propose that the PTX-sensitive activity is caused by spontaneous release of GABA from inhibitory GABAergic inputs to these neurons.

We have also recorded synaptic currents from neurons isolated from the cerebral cortex and from other regions of the medulla. Biophysical experiments on natural synaptic transmission are no longer limited to procedures requiring the development of synapses in vitro between cultured immature neurons.

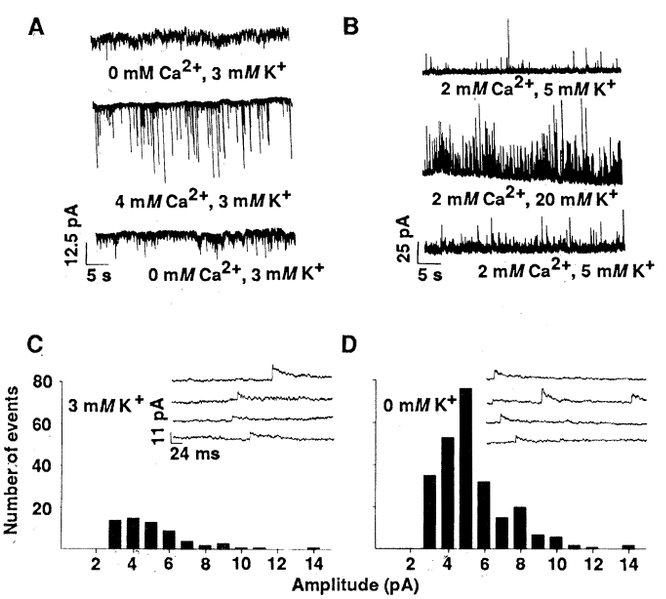

Fig. 4. Dependence of spontaneous events on extracellular Ca^{2+} or K^+ concentration. (**A**) Reversible increase in spontaneous excitatory postsynaptic activity with application of external Ca^{2+}; holding potential −80 mV. Neurons were dissociated in the external Hepes solution with 0 m*M* Ca^{2+} and 1 m*M* Mg^{2+}. Application of an external Hepes solution with 4 m*M* Ca^{2+} and 0 m*M* Mg^{2+} was by pressure ejection. (**B**) Reversible increase in activity with presynaptic terminal depolarization. Neurons were dissociated and bathed in a Hepes solution with 5 m*M* K^+ and clamped at +20 mV. Potassium was added to the bath to raise the bath K^+ concentration to 20 m*M*. The response was reversed by perfusion of the original bath solution. Amplitude histograms of mIPSC before (**C**) and after (**D**) synaptic terminal depolarization. The peak of the quantal mIPSC activity was ~4 pA, amplitudes larger than this being two to three times that of the mIPSC. (C) Neuron clamped at 0 mV for 365 s. (D) Same neuron clamped at 0 mV for 330 s after changing to 0 m*M* K^+ for bouton depolarization. Activity less than 3 pA in amplitude would not be distinguishable from random single openings of $GABA_A$ channels. Insets are representative traces showing mIPSC. Recordings were done in an external solution with 0 m*M* Ca^{2+} and 1.3 m*M* Mg^{2+}. The control K^+ concentration was 3 m*M* with terminal depolarization achieved by perfusing the neuron with 0 m*M* K^+ (*19*). Results were typical of (A) in three experiments, of (B) in three experiments, and of (C) and (D) in four experiments.

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Overexpression of Metallothionein Confers Resistance to Anticancer Drugs

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Resistance to antineoplastic agents is the major obstacle to curative therapy of cancer. Tumor cell lines with acquired resistance to the antineoplastic agent *cis*-diamminedichloroplatinum(II) overexpressed metallothionein and demonstrated cross-resistance to alkylating agents such as chlorambucil and melphalan. Human carcinoma cells that maintained high levels of metallothionein because of chronic exposure to heavy metals were resistant to *cis*-diamminedichloroplatinum(II), melphalan, and chlorambucil. Furthermore, cells transfected with bovine papilloma virus expression vectors containing DNA encoding human metallothionein-II_A were resistant to *cis*-diamminedichloroplatinum(II), melphalan, and chlorambucil but not to 5-fluorouracil or vincristine. Thus, overexpression of metallothionein represents one mechanism of resistance to a subset of clinically important anticancer drugs.

CIS-DIAMMINEDICHLOROPLATINUM (CP) is one of the most widely used antitumor agents, forming the cornerstone of chemotherapy of testicular, ovarian, and small cell lung carcinomas. As an electrophilic metal coordination complex, CP interacts with DNA to form bidentate adducts (*1*), thus exerting an effect similar to other therapeutically valuable antitumor alkylating agents, such as melphalan and chlorambucil. The development of resistance to antineoplastic agents significantly restricts their efficacy (*2*). Therefore, identification of the mechanisms of antineoplastic drug resistance is a critical precursor to the design of new treatment strategies for cancer. The development of resistance to CP or the alkylating agents has been explained by several factors, including reduced drug internalization, increased DNA repair processes, and an increase in inactivating proteins such as cellular thiols (*1, 3, 4*).

Metallothioneins (MTs) are cysteine-rich proteins that are present in a wide variety of eukaryotes and that constitute the major fraction of the intracellular protein thiols. They complex with group II_B metals and function extensively in Zn^{2+} and Cu^{2+} homeostasis and heavy metal detoxification (*5*). In addition, indirect evidence shows that the abundant nucleophilic sulfhydryl groups in MT can interact with many electrophilic toxins, participate in controlling intracellular redox potential, and act as scavengers of oxygen radicals generated during the metabolism of xenobiotics (*5*). The transcription of the MT gene family is controlled both by metals and by nonmetal environmental stimuli such as epinephrine, glucocorticoids, thermal injury, cytokines, cyclic nucleotides, and phorbol esters (*5*).

Metallothioneins are attractive candidates as modulators of cellular sensitivity to electrophilic anticancer agents (*6–10*), although no direct evidence to support their involvement has yet been reported. Several investigators (*9, 10*) have observed an increase in

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Table 1. Relation between MT content and drug resistance. The cell lines used were SCC-25, human head and neck carcinoma; G3361, human melanoma; SW2, human small cell carcinoma; SL6, human large cell carcinoma; A-253, human head and neck carcinoma; and L1210, murine lymphocytic leukemia. The drugs used were CP; Cd, $CdCl_2$; Blm, bleomycin; and DACH. Resistance ratio is defined as the ratio of CP concentration required to inhibit cellular proliferation by 50% (IC_{50}) in resistant cells to the IC_{50} of the parental cells. Cells in exponential growth were treated with various concentrations of CP; IC_{50} values of human tumor cell lines were determined as described (*9, 16*). We determined the IC_{50} for murine L1210 cells (in suspension) by counting the number of cells after they were treated with continuous exposure to various concentrations of CP for 3 days (*17*).

Cell line	CP resistance ratio	MT content* (fold increase ± SEM)
SCC-25/CP	7.1	4.37 ± 0.50
G3361/CP	6.7	2.00 ± 0.10
SW2/CP	4.5	5.10 ± 0.50
SL6/CP	2.5	3.37 ± 0.44
MCF-7/CP	2.5	0.95 ± 0.11
A-253/Cd	2.7	3.27 ± 0.37
A-253/C-10/Blm	0.88	0.72 ± 0.04
L1210/CP	44	13.3 ± 1.50
L1210/CP-R	5.8	2.18 ± 0.15
L1210/DACH	2.7	2.94 ± 0.18

*The MT content was estimated by an indirect competitive ELISA. Polyclonal rabbit antibody to rodent and human MT-I and MT-II showed reactivity at 1:3000 (*12*). Rabbit MT-I and MT-II (50 ng/ml) were fixed to microtiter plates overnight at 4°C. Unbound sites were blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Cytosolic extracts from tumor cells or known amounts of competitor MT as standards were incubated with the rabbit antiserum and then applied to the microtiter wells. After 2 to 6 hours at 37°C and washing with PBS–Tween 20 (0.05%), a goat antibody to rabbit immunoglobulin G complexed with horseradish peroxidase (HyClone) was added at 1:3000. After incubation at 23°C for 1 hour and three washings, *o*-phenylenediamine (0.4 mg/ml) containing 0.1% urea peroxide in 0.1*M* citrate buffer, *p*H 4.5, was added as substrate. Color development was monitored by ELISA reader at 492 nm. Standard curves were generated with logit methodology (*18*). MT content (nanograms of MT per microgram of protein) (mean ± SE) of the parental lines were SCC-25, 0.46 ± 0.03; G3361, 0.39 ± 0.027; SW2, 0.052 ± 0.002; SL6, 0.115 ± 0.029; MCF-7, 0.09 ± 0.004; A-253, 1.3 ± 0.6; and L1210, 0.46 ± 0.038. Protein content of cytosolic extract was determined by the method of Bradford (*19*). The fold increase in MT was calculated as the ratio of MT in resistant cell lines to that in parental cell lines. Results were from three or more experiments.