0026-895X/90/010102-12$02.00/0

MOLECULAR PHARMACOLOGY, 38:102-113

Characterization of Interactions of Methylmercury with Ca^{2+} Channels in Synaptosomes and Pheochromocytoma Cells: Radiotracer Flux and Binding Studies

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Received October 2, 1989; Accepted April 30, 1990

SUMMARY

The interaction of methylmercury (MeHg) with neuronal Ca^{2+} channels in rat forebrain synaptosomes and dihydropyridine (DHP)-sensitive Ca^{2+} channels in rat pheochromocytoma (PC12) cells was examined using radiotracer flux assays and radioligand binding analyses. In synaptosomes, the influx of $^{45}Ca^{2+}$ was used to examine the voltage and state dependence of block of Ca^{2+} channels by MeHg, as well as the effects of MeHg on apparent inactivation of $^{45}Ca^{2+}$ influx. In addition, the differential influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$ was used to examine the effect of MeHg on the ionic selectivity of synaptosomal Ca^{2+} channels. The ability of MeHg to block $^{45}Ca^{2+}$ influx via a DHP-sensitive Ca^{2+} channel was examined in PC12 cells. Effects of MeHg on binding of [^{3}H]nitrendipine in synaptosomes and ^{125}I-ω-conotoxin GVIA (CgTx) in synaptosomes and PC12 cells were measured. In synaptosomes, MeHg blocked $^{45}Ca^{2+}$ influx in a voltage-dependent manner, inasmuch as increasing the extracellular K^+ concentration increased the magnitude of block by 100 μM MeHg. When synaptosomes were incubated for 10 sec in either a nondepolarizing or a depolarizing solution before measurement of 1 sec of depolarization-induced $^{45}Ca^{2+}$ influx, the potency and efficacy of the block of $^{45}Ca^{2+}$ influx by MeHg were similar. Thus, block of Ca^{2+} channels by MeHg does not appear to be state dependent. To determine the kinetics of apparent inactivation of $^{45}Ca^{2+}$ influx, synaptosomes were predepolarized in Ca^{2+}-free high [K^+] solution, for intervals varying from 1 to 10 sec, before measurement of 1 sec of K^+-induced $^{45}Ca^{2+}$ influx. When compared with control, MeHg (100 μM) altered the rate constant for apparent inactivation and decreased the fraction of $^{45}Ca^{2+}$ influx that does not inactivate. Influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$ during 1 sec of depolarization was blocked in a dose-dependent manner by MeHg, with estimated IC_{50} values of 125, 150, and >150 μM for $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$, respectively. In triple-label experiments, the relative flux of radiolabeled Ca^{2+}:Sr^{2+}:Ba^{2+} was altered from approximately 6:2:3 to 6:1:3 in the presence of 100 μM MeHg. In undifferentiated and nerve growth factor-differentiated PC12 cells, K^+-induced $^{45}Ca^{2+}$ influx was blocked by the DHP nifedipine, with an approximate IC_{50} value of 5 nM. MeHg reduced $^{45}Ca^{2+}$ influx in PC12 cells with an estimated IC_{50} value of 50 μM, and 125 μM MeHg reduced uptake by >90%. [^{3}H]Nitrendipine bound to synaptosomes with high affinity in normal and elevated [K^+] solutions. In normal [K^+] solutions, Scatchard analysis of the binding data resulted in a K_D value of 630 ± 160 pM and a B_{max} value of 130 ± 40 fmol/mg of protein. In the presence of 100 μM MeHg, the values for K_D and B_{max} were 2520 ± 630 pM and 200 ± 30 fmol/mg of protein, respectively. Results obtained in the absence and presence of MeHg in elevated [K^+] buffers were not significantly different from those obtained in normal [K^+] buffers. ^{125}I-CgTx bound to a single high-affinity site on synaptosomes and PC12 cells, with half-saturation occurring in the subnanomolar range. MeHg at 100 μM did not significantly alter binding of CgTx in synaptosomes but did alter CgTx binding in PC12 cells by decreasing the amount of CgTx bound at all concentrations of CgTx tested. These results indicate that MeHg 1) blocks synaptosomal Ca^{2+} channels in a voltage- but not state-dependent manner, 2) alters synaptosomal Ca^{2+} channel ionic selectivity and inactivation kinetics, 3) blocks Ca^{2+} influx via DHP-sensitive Ca^{2+} channels in PC12 cells, and 4) alters the binding properties of DHPs in synaptosomes and of CgTx in PC12 cells but not synaptosomes.

MeHg, an organic monovalent metal, disrupts synaptic transmission (1, 2) and exerts prominent neurotoxic effects following acute or chronic exposure (3). The characteristic disruption of synaptic transmission produced by MeHg during acute administration appears to be due in part to block of depolarization-dependent entry of Ca^{2+} into the axon terminal (2, 4, 5), thereby undermining this critical step in the release of chemical neurotransmitters (6, 7). This blocking action of MeHg has been

This work was supported by National Institutes of Health Grant ES03299. Preliminary results were presented at the 18th and 19th Annual Meetings of the Society for Neuroscience, November 13-18, 1988, in Toronto, Ontario, Canada, and October 29 to November 3, 1989, in Phoenix, AZ, respectively, and the 28th Annual Meeting of the Society of Toxicology, February 27 to March 3, 1989, in Atlanta, GA, and published in abstract form (71-73). This paper was submitted by T.J.S. in partial fulfillment of the requirements for the Ph.D. degree in Pharmacology and Toxicology. W.D.A. is supported by a Research Career Development Award, 1 K04 ES00178. T.J.S. is the recipient of a Student Fellowship sponsored by Hoffman-LaRoche, Inc.

ABBREVIATIONS: MeHg, methylmercury; DHP, dihydropyridine; CgTx, ω-conotoxin GVIA; EGTA, ethyleneglycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; ANOVA, analysis of variance; NGF, nerve growth factor; LSD, least significant difference.

studied in isolation using the synaptosome, a model system of nerve terminals derived from the mammalian central nervous system. Micromolar concentrations of MeHg (8) or inorganic divalent cations (Hg^{2+}, Pb^{2+}, and Cd^{2+}) (4, 9) block 1 sec ("fast" phase) of depolarization-induced influx of $^{45}Ca^{2+}$ into synaptosomes. Increasing the extracellular Ca^{2+} concentration readily reverses block of fast influx by divalent cations (9), but not by MeHg (4, 8). The fast phase of $^{45}Ca^{2+}$ influx into synaptosomes is clearly associated with neurotransmitter release (10–12) and is thought to be mediated by voltage-dependent Ca^{2+} channels.

MeHg differs in two important respects from traditional divalent inorganic Ca^{2+} channel blockers, enhanced lipophilicity imparted by the methyl group and monovalent rather than polyvalent charge. Because data suggest that MeHg disrupts Ca^{2+} entry into the nerve terminal by interacting directly with Ca^{2+} channels and because of the chemical differences between MeHg and other heavy metal Ca^{2+} channel blockers, experiments were designed to characterize in detail the nature of the interaction of MeHg with nerve terminal Ca^{2+} channels. Specifically, experiments were designed to determine whether MeHg blocks Ca^{2+} channels in a voltage- and/or state-dependent manner and whether MeHg is capable of altering the kinetics of apparent inactivation and/or ionic selectivity of synaptosomal Ca^{2+} channels.

The observation that multiple types of Ca^{2+} channels exist in nerve terminals (13–15) raises the question of the identity of Ca^{2+} channels that are blocked by MeHg. Because the type or types of Ca^{2+} channels in synaptosomal preparations are not well characterized, rat pheochromocytoma (PC12) cells were chosen as an alternative model system for studies of the effects of MeHg on Ca^{2+} channels. PC12 cells are a clonal cell line possessing several unique characteristics that can be utilized to study voltage-dependent Ca^{2+} channels. Under normal culture conditions, PC12 cells morphologically resemble adrenal chromaffin cells and express predominantly a DHP-sensitive Ca^{2+} channel (16) known as L- or "long opening"-type (17, 18). The addition of NGF to the culture medium results in the outgrowth of nerve processes from PC12 cells and, after approximately 7 days, the cells morphologically resemble sympathetic neurons (19). This process of differentiation is also associated with the expression of novel proteins (20) and increased channel activity (21, 22), including increases in a DHP-insensitive Ca^{2+} conductance (23). This DHP-insensitive Ca^{2+} conductance has been characterized electrophysiologically using patch voltage-clamp analysis (24) and resembles the N-type of Ca^{2+} conductance described in other systems (17, 18, 25). Thus, PC12 cells provide a means by which to examine the effects of MeHg on a homogeneous and/or defined population of Ca^{2+} channels.

Finally, the effects of MeHg on the binding of two well characterized Ca^{2+} channel antagonists were examined. Although the pharmacological binding potency of Ca^{2+} channel antagonists does not necessarily correlate with their functional effects, competitive binding assays, nevertheless, provide a measure of the ability of MeHg to compete with known Ca^{2+} channel antagonists for their binding sites on nerve membranes. The DHP nitrendipine has been employed in numerous binding and flux studies in synaptosomes (26–28) and PC12 cells (29) to examine the properties of binding sites that are presumed to be located on or associated with L-type Ca^{2+} channels. Therefore, the effects of MeHg on the binding of [^{3}H] nitrendipine could provide additional information regarding the ability of MeHg to interact with binding sites associated with this type of Ca^{2+} channel.

CgTx, a peptide isolated from the venom of the marine snail *Conus geographus*, reduces Ca^{2+} uptake in chick synaptosomes (30) and binds to sites in synaptosomes and PC12 cells that appear to be distinct from the DHP binding site (31–34). Therefore, the effects of MeHg on binding of CgTx were examined in synaptosomes and PC12 cells to determine whether MeHg may interact with these sites instead of, or as well as, DHP binding sites. Inasmuch as CgTx has been reported to inhibit transmitter release from and $^{45}Ca^{2+}$ influx into isolated nerve endings and to bind at distinct sites from the DHPs, its use would permit a differential analysis of binding of MeHg to sites spatially associated with perhaps two different Ca^{2+} channels or to two separate binding sites on Ca^{2+} channels.

Materials and Methods

Chemicals and solutions. Methylmercuric acetate was obtained from Pfaltz and Bauer, Inc. (Stamford, CT) and was dissolved in 4% (v/v) glacial acetic acid to make a 2 mM stock solution. Aliquots of this solution were diluted in the appropriate buffers and adjusted to pH 7.4 at room temperature (25°) to yield the final concentrations of MeHg indicated in the text after the addition of synaptosomes or PC12 cells. *N*-Methylglucamine, EGTA, polyethyleneimine, nifedipine, and choline chloride were obtained from Sigma Chemical Co. (St. Louis, MO). HEPES was purchased from United States Biochemical Corporation (Cleveland, OH). Radioisotopes were purchased from New England Nuclear Co. (Boston, MA). Unlabeled CgTx was purchased from Peninsula Laboratories (Belmont, CA).

For incubation of synaptosomes, physiological saline contained (mM): NaCl, 145; KCl, 5; $MgCl_2$, 1; D-glucose, 10; and HEPES, 10. To depolarize synaptosomes, elevated [K^+] solution contained (mM): NaCl, 72.5; KCl, 77.5; $MgCl_2$, 1; $CaCl_2$, 0.04; D-glucose, 10; and HEPES, 10. Normal [K^+] (5 mM) solution contained the same constituents and amounts as did elevated [K^+] solution, except that [K^+] was lowered to 5 mM by replacement of KCl with 72.5 mM choline chloride. Ca^{2+}-free solutions contained no added $CaCl_2$ but may have contained residual amounts of Ca^{2+} (1–2 μM). For experiments requiring Na-free solutions, NaCl was replaced by choline chloride on an equimolar basis in all solutions, including physiological saline. Quenching solution contained (mM): KCl, 5; $MgCl_2$, 2; EGTA, 1; D-glucose, 10; HEPES, 10; and *N*-methylglucamine, 145. *N*-Methylglucamine, which replaces Na^+ in the buffer, does not readily permeate the voltage-dependent sodium channel (35). All solutions were adjusted to pH 7.4 at room temperature (25°). Elevated (100 mM) and normal (5 mM) [K^+] PC12 medium used in influx studies with PC12 cells have been described by Stallcup (36) and are similar to those described above.

Synaptosomal experiments. Synaptosomes were prepared from forebrains of male Sprague-Dawley rats (Harlan, 175–250 g), using a modification of the method of Gray and Whittaker (37) described previously in detail (4, 8, 38). After centrifugation on sucrose density gradients, synaptosomes were washed and resuspended in physiological saline and incubated at 37° for 20 min before the experiment, except for binding experiments, for which synaptosomes were allowed to acclimate to room temperature for 25 min. The use of sucrose density gradients greatly reduces contamination by mitochondria and endoplasmic reticulum, which may influence the results of flux and binding assays.

For all subsequent experiments, the methods of Nachshen and Blaustein (39, 40) were used to measure influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, or $^{133}Ba^{2+}$ (specific activity, 15–47, 10.7, and 5.8 mCi/mg, respectively) into synaptosomes. Influx of label was measured by the addition of 50 μl of synaptosomal suspension to 50 μl of elevated (77.5 mM, except for K^+-dependence experiments) or normal (5 mM) [K^+] solution containing 0.06 mM label, unless otherwise noted. Influx was stopped after

various intervals of time by addition of 2 ml of quenching solution. For intervals up to 10 sec, an electronic metronome was used to time the addition of solutions, whereas a stopwatch was employed to time the addition of solutions after longer intervals. After addition of quenching solution, synaptosomes were filtered rapidly using Millipore filters (0.45 μm) and then washed with two 5-ml aliquots of quenching solution. Radioactivity was determined by liquid scintillation counting in a Searle Mark III scintillation counter with a 70% efficiency for $^{45}Ca^{2+}$ or in a Searle model 1197 γ-counter with efficiencies of 47 and 53% for $^{85}Sr^{2+}$ and $^{133}Ba^{2+}$, respectively. In all experiments except for K^+-dependence experiments, radiotracer influx in normal [K^+] solutions was later subtracted from that obtained in elevated [K^+] solutions to calculate net depolarization-induced influx, which is expressed as a function of synaptosomal protein content (41).

The K^+ dependence of $^{45}Ca^{2+}$ influx was measured in the presence and absence of MeHg (100 μM) by incubation of synaptosomes for 2 sec in Na-free K^+ solutions containing $^{45}Ca^{2+}$. Choline chloride in normal [K^+] solution was replaced by KCl to achieve the various K^+ concentrations used. To obtain a more accurate estimate of influx at the lower K^+ concentrations used, influx was measured for 2 sec at all K^+ concentrations.

The state dependence of block of the fast phase of $^{45}Ca^{2+}$ influx by MeHg was determined by measuring 1 sec of $^{45}Ca^{2+}$ influx in synaptosomes that had been exposed to MeHg under conditions in which the population of Ca^{2+} channels would be primarily in the "resting" state or under conditions in which the population of Ca^{2+} channels would be primarily in the "open" or "inactivated" state. To open or inactivate Ca^{2+} channels, synaptosomes were predepolarized in 41.25 mM K^+ solution for 10 sec by addition of 25 μl of synaptosomal suspension (5 mM K^+) to 25 μl of elevated [K^+] (77.5 mM) solution containing MeHg. Ca^{2+} channels in the resting state were exposed to MeHg in 5 mM K^+ solution for 10 sec by addition of 25 μl of synaptosomes (5 mM K^+) to 25 μl of normal (5 mM) [K^+] solution containing MeHg. Following the 10-sec exposure to MeHg, 1 sec of $^{45}Ca^{2+}$ influx was measured by addition of 50 μl of K^+ solution (77.5 or 113 mM K^+) containing 0.06 mM $^{45}Ca^{2+}$ and MeHg, so that the final K^+ concentration of all solutions was equal to 59.4 mM. One second after the addition of K^+ solution, $^{45}Ca^{2+}$ influx was stopped by the addition of 2 ml of quenching solution. In order to maintain osmolarity conditions equal to those in other experiments and to increase [K^+] to 113 mM, [Na^+] was lowered to 36 mM in K^+ solutions, being replaced by KCl (113 mM K^+ solutions) or choline chloride (physiological saline and 77.5 mM K^+ solutions) on an equimolar basis.

The apparent inactivation of depolarization-induced Ca^{2+} influx was determined by measuring 1 sec of $^{45}Ca^{2+}$ influx after various intervals of predepolarization (0–10 sec) in nominally Ca^{2+}-free medium to inactivate Ca^{2+} channels (28). Briefly, 200 μl of Ca^{2+}-free elevated (77.5 mM) [K^+] solution, with or without MeHg, were combined with 200 μl of synaptosomal suspension (5 mM K^+). Following 1 to 10 sec of predepolarization, 400 μl of elevated [K^+] solution containing 0.02 mM $^{45}Ca^{2+}$ and MeHg were added to the mixture. After an additional 1 sec, 3 ml of ice-cold quenching solution were added to stop $^{45}Ca^{2+}$ influx. The final concentration of MeHg before the addition of quenching solution was 100 μM. Noninactivated influx (1 sec) was measured by combining 200 μl of synaptosomal suspension with 600 μl of elevated [K^+] solution containing MeHg (100 μM) and $^{45}Ca^{2+}$. Synaptosomes were retained on Millipore filters and $^{45}Ca^{2+}$ influx was determined as described above.

PC12 cell experiments. PC12 cells were cultured at 37° in a humidified 5% CO_2 atmosphere, using Dulbecco's modified Eagle medium (pH 7.4, GIBCO) containing 10% horse serum, 5% fetal bovine serum, and 2 mM HEPES. PC12 cells were differentiated by culturing for 7 days in medium containing 100 ng/ml NGF isolated from mouse submaxillary gland. The NGF was a gift from Dr. Steven Heidemann at Michigan State University.

Cells were harvested by suspension in normal (5 mM) [K^+] PC12 medium and centrifugation for 10 min at 5000 × *g*. This step was repeated twice to wash the growth medium completely from the cells.

The effects of nifedipine and MeHg on depolarization-induced $^{45}Ca^{2+}$ influx were measured using the same method as for synaptosomes, except that elevated [K^+] PC12 medium contained 100 mM K^+ so that the final concentration of K^+ used for depolarization of the PC12 cells was 52.5 mM. Influx was measured over 2 min. PC12 cells were not preincubated with nifedipine or MeHg; therefore, the only exposure of the PC12 cells to MeHg or nifedipine was the 2 min during which $^{45}Ca^{2+}$ influx was measured. The final concentration of Ca^{2+} was 1 mM. During filtrations, the cells were retained on Whatman GF/B glass fiber filters.

Binding experiments. The equilibrium binding of [^{3}H]nitrendipine (specific activity, 70 Ci/mmol) was measured in synaptosomes in the following manner. For any given experiment, 2 ml of a stock solution containing [^{3}H]nitrendipine and MeHg (100 μM), when appropriate, were prepared in normal [K^+] solution. Binding of [^{3}H]nitrendipine was initiated by addition of 20 μl of synaptosomal suspension (250–350 mg of protein) to 500 μl of stock solution. After 1 hr of incubation at 25°, the solution was filtered rapidly through Whatman GF/B glass fiber filters and rinsed with three 5-ml aliquots of quenching solution. To determine total ligand concentration, a 50-μl aliquot was taken from the remaining 500 μl of stock solution. Nonspecific binding was measured in the presence of 1 μM nifedipine. Radioactivity remaining on filters was determined as described for $^{45}Ca^{2+}$ influx experiments, except that the scintillation counter contained a ^{3}H quench curve and a program to convert cpm to dpm. The averages of triplicate values were used for Scatchard analysis (42) to determine the equilibrium dissociation constant (K_D) value for binding of [^{3}H]nitrendipine and the density of binding sites (B_{max}). As with all experiments using DHPs, these procedures were carried out in the dark. To measure the binding of [^{3}H]nitrendipine under depolarizing conditions, exactly the same procedure was followed, using elevated [K^+] solution in place of normal [K^+] solution.

Binding of ^{125}I-CgTx (specific activity, 2200 Ci/mmol) to PC12 cells and synaptosomes was measured under nondepolarizing conditions by methods similar to those described for nitrendipine, except that PC12 cell medium was used for binding experiments in PC12 cells. In addition, the effects of pretreatment of synaptosomes with MeHg on CgTx binding were examined in the following manner. Once isolated, synaptosomes were resuspended in 5 ml of physiological saline containing 100 μM MeHg and were incubated at room temperature for 10 min. Following the incubation, synaptosomes were repelleted and washed in MeHg-free physiological saline by centrifugation at 10,000 × *g* for 10 min and subsequent resuspension. Synaptosomes were then incubated for 25 min at room temperature (25°) and 10-μl aliquots were used to measure the binding of ^{125}I-CgTx, as described above for nitrendipine. In all CgTx binding experiments, nonspecific binding was measured in the presence of 1 μM unlabeled CgTx and normal [K^+] solution contained 0.1% albumin and no added calcium. After 1 hr, solutions were filtered rapidly over Whatman GF/B filters that had been presoaked in quenching solution containing 0.1% polyethyleneimine. Radioactivity remaining on the filters was determined in the Searle model 1197 γ-counter with an efficiency of 53% for ^{125}I. Protein content of synaptosomal and PC12 cell suspensions was determined by the method of Lowry *et al.* (41).

Statistical analysis. Statistical analysis of results of the inactivation experiments is described in Results. For experiments using nifedipine or MeHg to block $^{45}Ca^{2+}$ influx into PC12 cells or to measure the time-course of block by MeHg in synaptosomes, data were analyzed using a randomized block ANOVA and, if significant differences were found, individual comparisons were made using Dunnett's one-tailed *t* test. For K^+-dependence experiments, a mixed design ANOVA was used to compare influx in control and MeHg-treated synaptosomes; individual comparisons were made using the LSD test. To compare the efficiency of block by MeHg at different [K^+], a randomized block ANOVA and Dunnett's one-tailed *t* test were used. Data from triple-label studies were analyzed using a randomized block ANOVA; individual comparisons were made using Scheffe's test. Comparisons of K_D values for [^{3}H]nitrendipine binding to synaptosomes were made using a mixed design ANOVA and the LSD test. Data from ^{125}I-CgTx binding experiments were analyzed using a randomized complete block ANOVA

and the LSD test. For all comparisons, $P < 0.05$ was considered significant.

Results

Time course of block by MeHg. Block of $^{45}Ca^{2+}$ influx into synaptosomes by MeHg as a function of time is shown in Fig. 1. MeHg (50 μM) blocked approximately 50% of the $^{45}Ca^{2+}$ influx during the first 1 sec of depolarization. There was no statistically significant change in the degree of block by MeHg as a function of time. The influx of $^{45}Ca^{2+}$ into synaptosomes consists of two distinct phases (28, 39), a fast phase, which is mediated by voltage-dependent Ca^{2+} channels, and a slow phase, which is mediated largely by reversed Na^+/Ca^{2+} exchange but may also contain a residual channel-mediated component (8). Because low concentrations of MeHg may not affect reversed Na^+/Ca^{2+} exchange in nerve terminals (8, 43), uptake via this system may offset the block by MeHg of channel-mediated influx. Therefore, the time-dependent effects of MeHg were examined in medium in which NaCl was replaced by choline chloride on an equimolar basis to prevent uptake via reversed Na^+/Ca^{2+} exchange. Under these conditions, block of $^{45}Ca^{2+}$ influx by MeHg increased as time of exposure during depolarization increased. After 10 or more sec of exposure to 50 μM MeHg, block of $^{45}Ca^{2+}$ influx was significantly greater than after only 1 sec of exposure (Fig. 1). Thus, as the time of exposure to MeHg increased, MeHg blocked a greater portion of channel-mediated depolarization-induced $^{45}Ca^{2+}$ influx.

K^+ (voltage)-dependence experiments. In Fig. 2A, the K^+-dependence of $^{45}Ca^{2+}$ influx into synaptosomes is shown in the absence and presence of 100 μM MeHg. Influx in 5 mM K^+ has not been subtracted from these data. The magnitude of depolarization caused by each K^+ concentration can be estimated by the relationship $\Delta V_m = 60 \log [K^+]_H/[K^+]_L$, where ΔV_m is the change in membrane potential and $[K^+]$ is the K^+ concentration of the normal (L) and elevated (H) $[K^+]$ solutions (44). Thus, the voltage dependence of influx can be estimated by this method. The K^+ concentrations used in this experiment will result in depolarizations of 0 to 55 mV from the resting membrane potential, which under similar conditions has been estimated to be approximately −80 mV (28). In the absence of MeHg, synaptosomal $^{45}Ca^{2+}$ influx activated at $[K^+]$ above 10 mM and increased in a linear manner as $[K^+]$ was increased to 41.3 mM. In a separate experiment, higher concentrations did not increase $^{45}Ca^{2+}$ influx further (data not shown). Thus, $^{45}Ca^{2+}$ influx in synaptosomes activates at K^+ concentrations above 10 mM and is maximal at K^+ concentrations of approximately 41 mM. These results are consistent with those of Suszkiw *et al.* (45). In the presence of 100 μM MeHg, $^{45}Ca^{2+}$ influx increased only gradually as the $[K^+]$ was increased, being significantly less than control values at each respective K^+ concentration. In Fig. 2B, the percentage of inhibition of $^{45}Ca^{2+}$ influx by MeHg is plotted as a function of $[K^+]$. At low K^+ concentrations, MeHg blocked approximately 20% of the $^{45}Ca^{2+}$ influx. As the $[K^+]$ was increased, block of uptake by 100 μM MeHg increased to approximately 50% at the highest K^+ con-

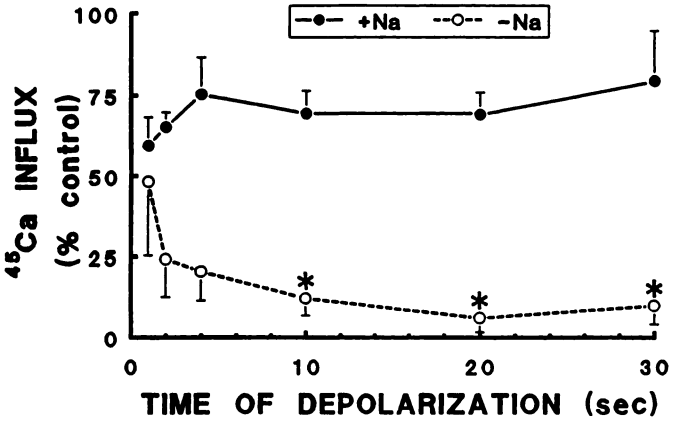

Fig. 1. Block of $^{45}Ca^{2+}$ influx into synaptosomes by MeHg as a function of time of exposure in Na-containing (●) or Na-free (○) medium. $^{45}Ca^{2+}$ influx was measured by the addition of 50 μl of synaptosomes to 50 μl of elevated (77.5 mM) or normal (5 mM) $[K^+]$ solutions containing $^{45}Ca^{2+}$ and 50 μM MeHg. Influx was stopped after various intervals of time by the addition of 2 ml of quenching solution. Influx in normal $[K^+]$ solutions was subtracted to determine depolarization-induced $^{45}Ca^{2+}$ influx. The results are expressed as a percentage of $^{45}Ca^{2+}$ influx in MeHg-free solutions and are the mean ± standard error of three or four separate experiments, respectively. Values for any particular experiment are the average of three replicates. *, Values that are significantly ($P < 0.05$) less than the value at 1 sec in Na-containing or Na-free synaptosomes, respectively. After 30 sec of depolarization, the values for influx of $^{45}Ca^{2+}$ into synaptosomes in the absence of MeHg were 1.99 ± 0.58 and 7.85 ± 2.22 nmol/μg of protein × 10^{-4} in Na-free and Na-containing solutions, respectively.

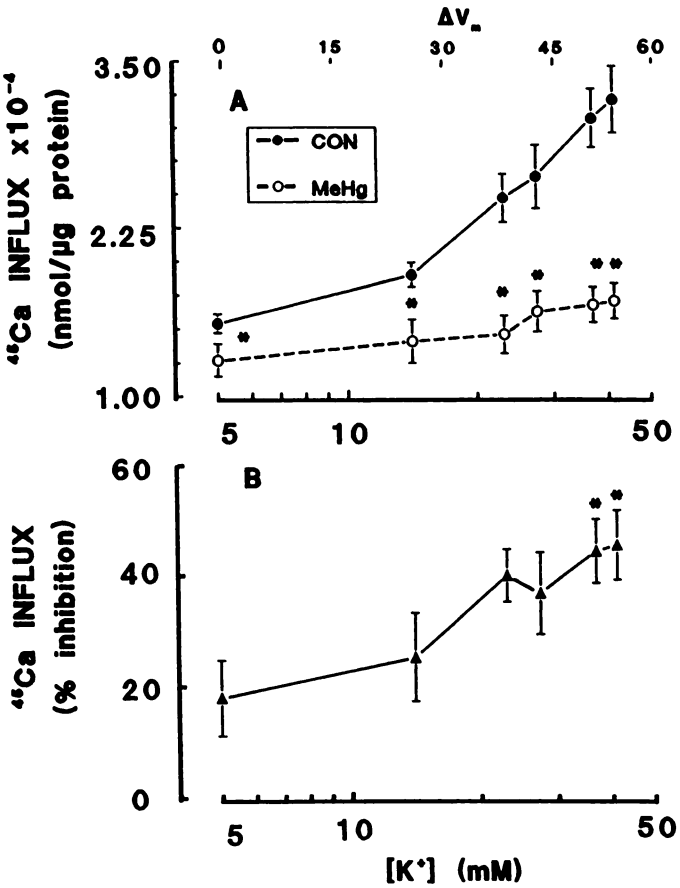

Fig. 2. A, Effects of $[K^+]$ on influx of $^{45}Ca^{2+}$ into synaptosomes in the absence (●) and presence (○) of 100 μM MeHg. Synaptosomes were incubated for 2 sec in Na-free buffers containing $^{45}Ca^{2+}$ and various concentrations of K^+. Influx was stopped by the addition of 2 ml of quenching solution. *, Values of influx in the presence of MeHg that are significantly ($P < 0.05$) less than their respective MeHg-free control value. B, Magnitude of block of $^{45}Ca^{2+}$ influx by 100 μM MeHg at different K^+ concentrations used in A. *, Values that show significantly ($P < 0.05$) greater block by MeHg than the block in 5 mM K^+. All values are the mean ± standard error of four experiments, where each experimental value is the average of three replicates.

centration tested. The block of $^{45}Ca^{2+}$ influx in 36 mM or higher concentrations of K^+ was significantly greater than that observed in 5 mM K^+ solutions.

State-dependence experiments. Block of Ca^{2+} channels in excitable membranes frequently exhibits so-called state dependence, wherein the blocking affinity of the antagonist is elevated depending upon the configuration of the channel. Inasmuch as the blocking affinity of certain divalent cations for $^{45}Ca^{2+}$ influx into synaptosomes has been shown to be state dependent (46), we sought to determine whether the degree of block by MeHg depended on prior utilization of the channel. This was accomplished by incubating synaptosomes for 10 sec in the presence of MeHg in either 5 mM or 41.3 mM K^+ solutions, before measuring 1 sec of depolarization-induced $^{45}Ca^{2+}$ influx. These K^+ concentrations are below and above the threshold for activation of $^{45}Ca^{2+}$ influx into synaptosomes, respectively. Thus, in synaptosomes incubated in 41.3 mM K^+, MeHg had access to the open and inactivated states of the Ca^{2+} channel, whereas in synaptosomes incubated in 5 mM K^+ solution, MeHg had access to Ca^{2+} channels in the resting state. After incubation in 5 mM K^+ solution, MeHg caused a dose-dependent suppression of 1 sec of $^{45}Ca^{2+}$ influx, which reached 84.5 ± 5.0% (mean ± SE; six experiments) reduction with 250 μM MeHg; the estimated IC_{50} value was 75 μM (Fig. 3). When MeHg had access to open or inactivated Ca^{2+} channels before measurement of 1 sec of influx, a dose-dependent suppression of $^{45}Ca^{2+}$ influx was also observed (Fig. 3). The estimated IC_{50} value for MeHg was also 75 μM and inhibition was maximum (89.4 ± 4.5%, mean ± SE; five experiments) at 250 μM MeHg. Although $^{45}Ca^{2+}$ influx at low concentrations of MeHg was reduced to a greater extent in predepolarized synaptosomes than in nondepolarized synaptosomes, there was no significant difference in the reduction of $^{45}Ca^{2+}$ influx under the two conditions. Thus, block of $^{45}Ca^{2+}$ influx by MeHg was not altered when MeHg was allowed access to different states of the Ca^{2+} channel.

Inactivation experiments. Predepolarization of synaptosomes in the absence of added Ca^{2+} resulted in a time-dependent decrease in $^{45}Ca^{2+}$ influx into synaptosomes when Ca^{2+}-containing solutions were subsequently added (Fig. 4). This decrease is presumably due to voltage-dependent inactivation of synaptosomal Ca^{2+} channels. We sought to determine whether MeHg altered this process in a quantifiable manner by examining a model for apparent inactivation in synaptosomes: $Q = q_1e^{-k_it} + q_2$, where Q is the fraction of Ca^{2+} influx remaining after time t of predepolarization, k_i is the rate constant for apparent inactivation, and q_1 and q_2 are the fractions of Ca^{2+} influx that do and do not inactivate, respectively. This relationship has been shown previously (28) to describe the apparent inactivation of Ca^{2+} influx in synaptosomes under conditions that closely approximate those in our experiments. To ensure that our data are described accurately by this model, we examined the goodness of fit of our data to this model by nonlinear least squares regression analysis. For control experiments, correlation coefficient values ranged from 0.77 to 0.94. We also examined the goodness of fit of our data to several other models that might also describe inactivation, including a model for the exponential decay of a voltage-activated current (47) that has been used recently to describe the apparent inactivation of Ca^{2+} influx in synaptosomes incubated in Na^+-free medium (45). Correlation coefficient values for goodness of fit of our data to these other models were less satisfactory than those for the model chosen.

Using the CRUNCH statistical program, nonlinear least squares regression analysis was performed on the data from each individual experiment to determine k_i, q_1, and q_2. The average values for k_i, q_1, and q_2 for control and MeHg-treated synaptosomes are listed in Table 1 and yield the lines in Fig. 4 when used in the above function. Significant differences in k_i, q_1, and q_2 in MeHg-treated (three experiments) and control (five experiments) synaptosomes were tested for, using a one-

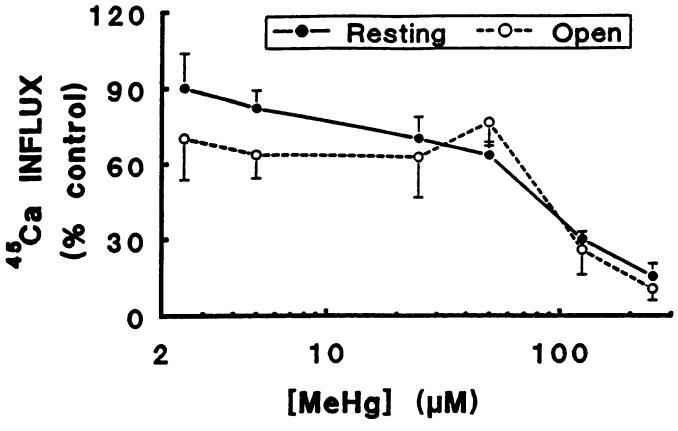

Fig. 3. Effects of exposure of synaptosomes to MeHg on 1 sec of $^{45}Ca^{2+}$ influx. Influx was measured during 1 sec of K^+-induced depolarization following predepolarization for 10 sec in the presence of MeHg (○) or following 10 sec of incubation with MeHg in a nondepolarizing solution (5 mM K^+) (●). Values are the mean ± standard error of six different experiments. For any given experiment, values are the average of three replicates.

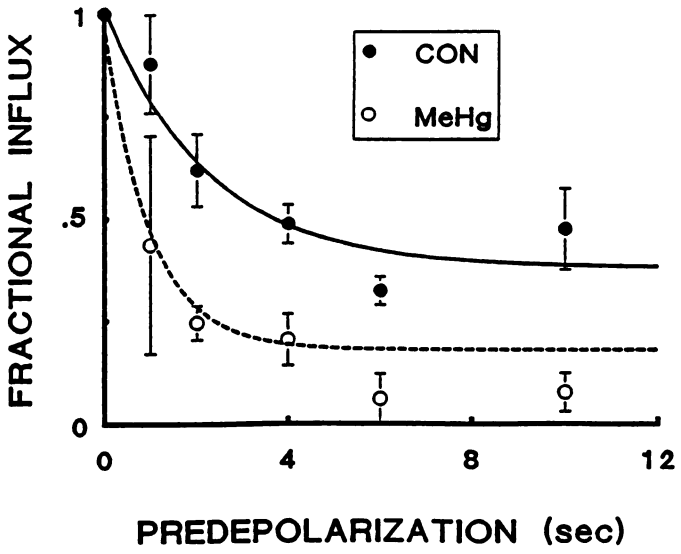

Fig. 4. Effects of MeHg (100 μM) on the rate of apparent inactivation of depolarization-induced $^{45}Ca^{2+}$ influx. Synaptosomes (200 μl) were predepolarized for varying intervals by addition of 200 μl of Ca^{2+}-free elevated [K^+] solution containing (○) or free of (●) MeHg. Following predepolarization, 400 μl of elevated [K^+] solution containing MeHg (if appropriate) and 1 μCi of $^{45}Ca^{2+}$ were added. Influx was stopped after 1 sec by the addition of 3 ml of quenching solution. Fractional influx is the amount of $^{45}Ca^{2+}$ influx remaining after a designated interval of predepolarization, relative to 1 sec of $^{45}Ca^{2+}$ influx in the absence of predepolarization. The values shown are the mean ± standard error of five (MeHg-free) or three (MeHg-containing) different experiments. Values for any particular experiment are the average of three replicates. *Curves* are drawn using the average nonlinear least squares values listed in Table 1.

sided Student's t test ($p \leq 0.05$). When MeHg-treated synaptosomes were compared with untreated control synaptosomes, the values for k_i, q_1, and q_2 were significantly different. Thus, MeHg alters the kinetics of apparent inactivation of $^{45}Ca^{2+}$ influx in synaptosomes by altering the rate of apparent inactivation and increasing the fraction of influx that inactivates. It should be noted that, in the presence of MeHg, inactivation of $^{45}Ca^{2+}$ influx was essentially complete after only 1 sec of predepolarization; thus, the value for the rate constant, k_i, only reflects the apparent constant for the rate of inactivation.

Ionic selectivity experiments. For dose-response curves, solutions contained a final concentration of 2.4 mM unlabeled Ca^{2+}, Sr^{2+}, or Ba^{2+} and 1 μCi of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, or $^{133}Ba^{2+}$, respectively. MeHg reduced depolarization-induced influx of $^{45}Ca^{2+}$ and $^{85}Sr^{2+}$ into synaptosomes in a dose-dependent manner, with estimated IC_{50} values of 125 and 150 μM, respectively (Fig. 5). Although the IC_{50} value for block of $^{45}Ca^{2+}$ uptake by MeHg is slightly higher than previously reported (8), the concentration of extracellular calcium used in this experiment was 2.4 mM, compared with 0.05 mM Ca^{2+} in previous experiments. In contrast to Ca^{2+} and Sr^{2+}, the influx of $^{133}Ba^{2+}$ was only slightly inhibited by concentrations of MeHg as high as 250 μM (Fig. 5). The differential sensitivity of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$ fluxes to block by MeHg suggests that MeHg alters the ionic selectivity of the Ca^{2+} channel for these ions. However, the decreased potency of MeHg to block Ba^{2+} influx may not be due to a reduced blocking action of MeHg on Ba^{2+} influx but to an increased influx of Ba^{2+} into synaptosomes due to lack of Ca^{2+}-induced Ca^{2+} channel inactivation by Ba^{2+} (48). In order to control for differences in Ca^{2+} channel inactivation, triple-label studies were designed in which the influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, or $^{133}Ba^{2+}$ into synaptosomes was measured in solutions that contained 0.1 mM Sr^{2+} and Ba^{2+}, 0.02 mM Ca^{2+}, and 0.5 mM Mg^{2+}. The effects of MeHg (100 μM) on the relative influx of the three divalent cations are shown in Fig. 6. To correct for differences in concentration, the values have been normalized by division of the influx of each ion by its concentration in the extracellular medium (40). In the absence of MeHg, the ratio of influx of ^{45}Ca:^{85}Sr:^{133}Ba was approximately 6:2:3. When 100 μM MeHg was present in the medium, the influx values for $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ were 67.1 ± 9.4 and 72.6 ± 11.6% of their respective control values, but the influx of $^{85}Sr^{2+}$ was only 44.1 ± 6.9% of its influx into synaptosomes in the absence of MeHg. This was a statistically significant difference when compared with the effects of MeHg on $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx into synaptosomes. Thus, MeHg altered the relative permeability of ^{45}Ca:^{85}Sr:^{133}Ba to 6:1:3.

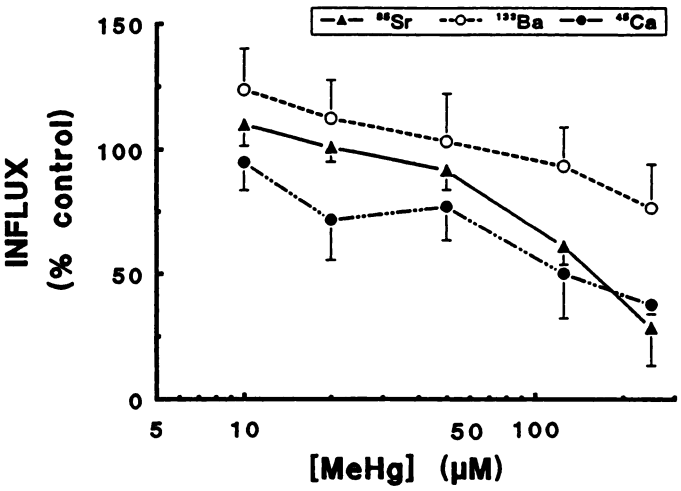

Fig. 5. Effects of MeHg on the influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$ into synaptosomes. Influx was measured by addition of 50 μl of synaptosomal suspension to 50 μl of elevated (77.5 mM) or normal (5 mM) [K^+] solution containing MeHg and 1 μCi of tracer. Influx was stopped after 1 sec by the addition of 2 ml of quenching solution. Depolarization-induced influx was determined by subtracting the influx of radiolabel in normal [K^+] solution. The values shown are the mean ± standard error of four ($^{45}Ca^{2+}$), three ($^{85}Sr^{2+}$), or five ($^{133}Ba^{2+}$) different experiments. Values for any particular experiment are the average of three replicates.

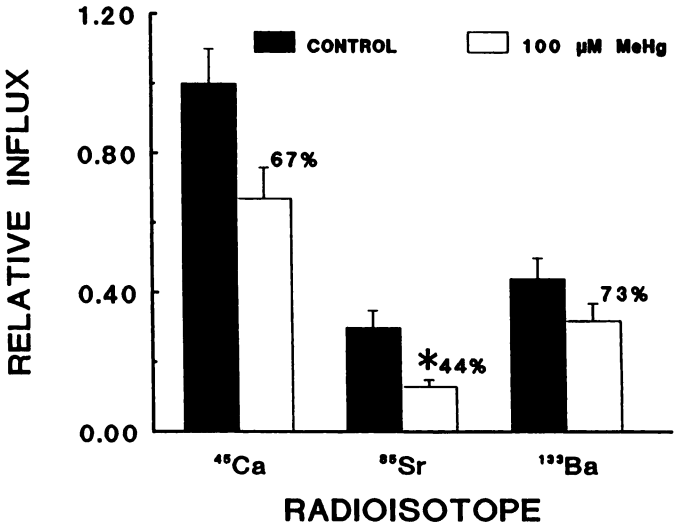

Fig. 6. Effects of MeHg on the relative influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$ into synaptosomes. Influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, or $^{133}Ba^{2+}$ during 1 sec of K^+-induced influx was measured in the absence (■) or presence (□) of 100 μM MeHg. K^+ solutions contained (mM): $SrCl_2$, 0.1; $BaCl_2$, 0.1; $CaCl_2$, 0.02; $MgCl_2$, 0.05; and 1 μCi of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, or $^{133}Ba^{2+}$. The results have been normalized by dividing the influx of each cation by its external concentration. The values shown are the mean ± standard error of three to five separate experiments. Values for any particular experiment are the average of three replicates. *, Effect of MeHg on $^{85}Sr^{2+}$ influx that is significantly different from the effect of MeHg on $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ influx ($P < 0.05$).

TABLE 1

Nonlinear least squares regression values for apparent inactivation of $^{45}Ca^{2+}$ influx into synaptosomes

Nonlinear least squares regression was used to fit data to the function $Q = q_1e^{-k_it} + q_2$, where k_i is the rate constant, q_1 is the fraction of influx that inactivates, and q_2 is the fraction of influx that does not inactivate.

	k_i	q_1	q_2
	sec^{-1}		
Control[a]	0.50 ± 0.10	0.66 ± 0.02	0.38 ± 0.04
MeHg[b]	6.33 ± 0.08[c]	0.74 ± 0.02[c]	0.26 ± 0.2[c]

[a] Data from five different experiments were used to calculate regression values, which are given as the mean ± standard error.

[b] Data from three different experiments using 100 μM MeHg were used to calculate regression values, which are given as the mean ± standard error.

[c] Significantly different from control values ($p \leq 0.05$).

$^{45}Ca^{2+}$ influx into PC12 Cells. Influx of $^{45}Ca^{2+}$ into PC12 cells by depolarization in 52.5 mM K^+ gradually increased as time of depolarization was increased, reaching a maximal level after about 1.5 to 2 min of depolarization (results not shown). The effects of nifedipine, a Ca^{2+} channel antagonist, on 2 min of $^{45}Ca^{2+}$ influx into PC12 cells are shown in Fig. 7. In two

experiments, the approximate IC_{50} value for nifedipine in non-differentiated PC12 cells was 5 nM, and 100 nM nifedipine reduced influx of $^{45}Ca^{2+}$ by greater than 95%. This is consistent with previous studies that have shown that Ca^{2+} influx in undifferentiated PC12 cells is largely DHP-sensitive (16, 29, 49); therefore, further repetitions were deemed unnecessary. In NGF-differentiated cells, nifedipine reduced depolarization-dependent $^{45}Ca^{2+}$ influx, with an estimated IC_{50} of approximately 5 nM (three experiments) (Fig. 7). When compared with untreated controls, nifedipine significantly reduced $^{45}Ca^{2+}$ influx at all concentrations tested in differentiated PC12 cells. Although $^{45}Ca^{2+}$ influx was largely DHP-sensitive, 10.4 ± 0.9% of control influx remained in cells treated with 100 nM nifedipine, possibly indicating a DHP-insensitive component. Higher concentrations of nifedipine were not tested.

In undifferentiated PC12 cells, MeHg reduced $^{45}Ca^{2+}$ influx with an IC_{50} of approximately 50 μM, and 250 μM MeHg blocked $^{45}Ca^{2+}$ influx completely (Fig. 8). In NGF-differentiated PC12 cells, nearly identical results were obtained (Fig. 8). MeHg concentrations of 50 μM and above significantly reduced $^{45}Ca^{2+}$ influx in undifferentiated and NGF-differentiated PC12 cells, when compared with untreated controls. Thus, MeHg blocks a DHP-sensitive Ca^{2+} channel in PC12 cells with nearly identical potency to its block of Ca^{2+} entry in synaptosomes. In addition, in two of three experiments, MeHg completely blocked $^{45}Ca^{2+}$ influx into NGF-differentiated PC12 cells. This may indicate an action of MeHg on a DHP-insensitive Ca^{2+} channel in PC12 cells but would require electrophysiological experiments to confirm. MeHg did not alter the depolarization-independent entry of $^{45}Ca^{2+}$ into undifferentiated or NGF-differentiated PC12 cells. In addition, concentrations of MeHg as high as 250 μM did not affect the viability of PC12 cells, as measured by trypan blue exclusion, even after 1 hr of exposure (data not shown). Thus, the effects of MeHg on $^{45}Ca^{2+}$ influx into PC12 cells appear to be due to disruption of voltage-dependent channel-mediated Ca^{2+} influx and not to cytotoxicity to PC12 cells.

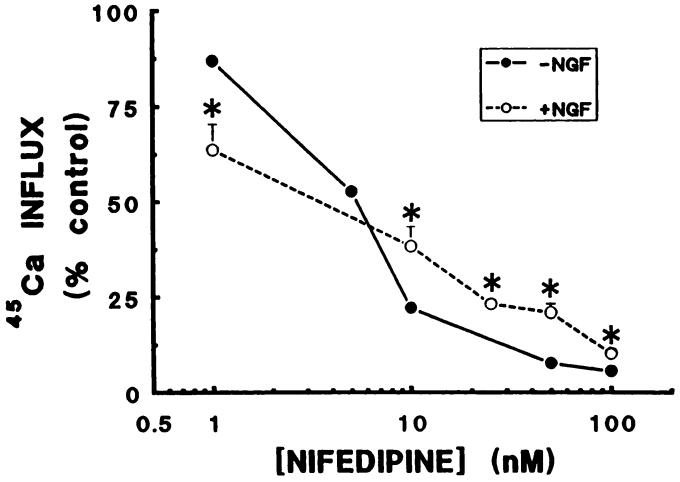

Fig. 7. Effects of nifedipine on depolarization-induced influx of $^{45}Ca^{2+}$ in undifferentiated (●) and NGF-differentiated (○) PC12 cells. Influx was measured by the addition of 50 μl of PC12 cell suspension in normal (5 mM) $[K^+]$ PC12 medium to 50 μl of elevated (100 mM) $[K^+]$ or normal $[K^+]$ PC12 medium containing 1 μCi of $^{45}Ca^{2+}$ and various concentrations of nifedipine. The values shown are the mean of two (nondifferentiated) or mean ± standard error of three (NGF-differentiated) different experiments. Values for any particular experiment are the average of three replicates. For experiments using NGF-differentiated cells, *error bars* are not shown if the standard error is smaller than the *symbol* size. *, Results that are significantly less than control ($P < 0.05$).

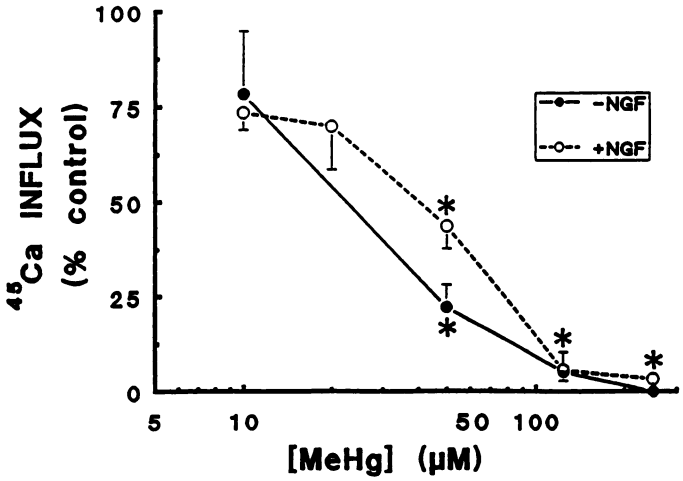

Fig. 8. Effects of MeHg on depolarization-induced $^{45}Ca^{2+}$ influx into undifferentiated (●) and NGF-differentiated (○) PC12 cells. $^{45}Ca^{2+}$ influx was measured as described in Fig. 7. The values shown are the mean ± standard error for three separate experiments. Values for any particular experiment are the average of three replicates. When *error bars* are not shown, the standard error is smaller than the size of the *symbol*. *, Results that are significantly less than control ($P < 0.05$).

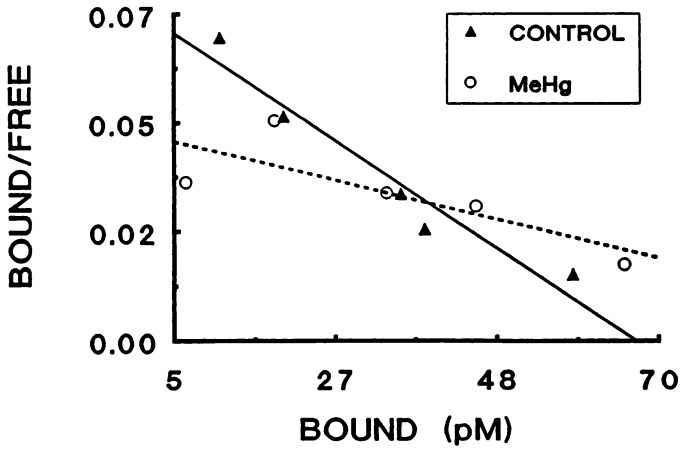

Fig. 9. Specific binding of $[^3H]$nitrendipine to synaptosomes in the absence (▲) and presence (○) of 100 μM MeHg in normal $[K^+]$ solutions. Values shown are the average of three replicates from a single representative experiment.

Nitrendipine binding. The binding of $[^3H]$nitrendipine to synaptosomes reached equilibrium after 10–15 min and was stable for 90 min (results not shown). When the binding of $[^3H]$nitrendipine (concentrations ranged from 0.18 to 3 nM) to synaptosomes was measured in normal (nondepolarizing) $[K^+]$ solutions, a single high affinity binding site was observed (Fig. 9). Nonspecific binding accounted for approximately 50% of total binding. Scatchard analysis of the data (three experiments) yielded a K_D value of 630 ± 160 pM and an apparent B_{max} value of 130 ± 40 fmol/mg of protein. The Hill slope was approximately equal to 1. These values are consistent with values reported by others (26–28) for $[^3H]$nitrendipine binding in synaptosomes. In the presence of 100 μM MeHg, the binding of $[^3H]$nitrendipine was also linear; however, the value for K_D was increased significantly over the control K_D value to 2520 ± 630 pM (three experiments). The apparent B_{max} was 200 ± 30

fmol/mg of protein in the presence of 100 μM MeHg. A valid determination of B_{max} could not be made due to the nitrendipine concentration range used; therefore, the B_{max} value obtained in the presence of MeHg is only an estimate and little direct information about the effects of MeHg on the number of binding sites can be gained by comparison with control B_{max} values. Under depolarizing conditions, similar results were obtained. In the absence of MeHg, the K_D value for the binding of [^{3}H]nitrendipine was 1100 ± 300 pM. Although the K_D was higher under depolarizing conditions, it was not significantly different from the K_D value in normal [K^+] solutions. In the presence of MeHg, the K_D value in elevated [K^+] solutions was 5330 ± 2100 pM and was significantly higher than the respective control value. Apparent B_{max} values in the absence and presence of 100 μM MeHg were 200 ± 23 and 320 ± 74 fmol/mg, respectively. Thus, MeHg decreased the affinity of binding of [^{3}H]nitrendipine to synaptosomes, indicating that it may act at or near the nitrendipine binding site. Because depolarization did not alter the characteristics of [^{3}H]nitrendipine binding to synaptosomes, the results of these experiments are reported here but are not illustrated in Fig. 9.

CgTx binding. The specific binding of ^{125}I-CgTx to synaptosomes in the absence and presence of MeHg is shown in Fig. 10A. Nonspecific binding accounted for approximately 20–30% of total binding. In the absence of MeHg, CgTx bound to synaptosomes with an apparent half-saturation of 0.1 to 0.2 nM. The maximum density of binding sites estimated by extrapolation of the saturated component to the *y*-axis is between 300 and 450 fmol/mg of protein. These results are similar to results for CgTx binding to synaptosomal membranes from rat and chick brain (31–33). In the presence of 100 μM MeHg, binding of CgTx to synaptosomes was not significantly different from that of control. Because the actions of MeHg at the neuromuscular junction (5) and in synaptosomes are not readily reversible (8), synaptosomes were pretreated with MeHg before measurement of CgTx binding. This pretreatment did not change the characteristics of CgTx binding (results not shown).

In undifferentiated PC12 cells, specific binding of ^{125}I-CgTx in the absence of MeHg was saturable (Fig. 10B). The apparent half-saturation was less than 0.1 nM CgTx. The apparent B_{max} was approximately 25–50 pmol/mg of protein. In the presence of 100 μM MeHg, the amount of ^{125}I-CgTx specifically bound was reduced significantly when binding was measured at CgTx concentrations between 10 and 500 pM. These results indicate that MeHg interacts with the receptor for CgTx on PC12 cells but not on synaptosomes.

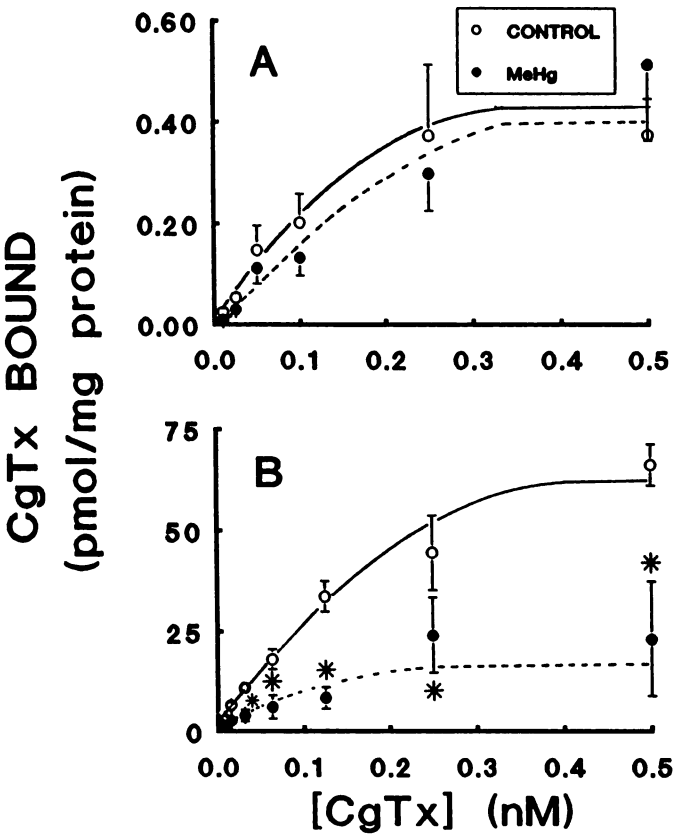

Fig. 10. Specific binding of ^{125}I-CgTx to synaptosomes (A) and PC12 cells (B) in the absence (○) and presence (●) of 100 μM MeHg. Values shown are the mean ± standard error of three experiments, where the values for any given experiment are the average of three replicates. When *error bars* are not shown, the standard error is smaller than the size of the *symbol*. *, Values that are significantly different from their respective MeHg-free control ($P < 0.05$).

Discussion

The goal of these experiments was to characterize the effects of MeHg on nerve terminal Ca^{2+} channel properties, using synaptosomes, and to attempt to identify the type or types of neuronal Ca^{2+} channels with which MeHg interacts in a defined population, using PC12 cells. Our results suggest that MeHg 1) blocks $^{45}Ca^{2+}$ influx into synaptosomes in a voltage-dependent manner, 2) blocks Ca^{2+} influx through synaptosomal Ca^{2+} channels in a manner that does not depend on the configuration (open, resting, or inactivated) of the Ca^{2+} channel, 3) acts on synaptosomal Ca^{2+} channels to alter both the kinetics of apparent inactivation and ionic selectivity, 4) is capable of blocking a DHP-sensitive Ca^{2+} channel in PC12 cells and may also block a DHP-insensitive Ca^{2+} channel in NGF-differentiated PC12 cells, and 5) alters nitrendipine and CgTx binding to high affinity sites on synaptosomes and PC12 cells, respectively.

The block of $^{45}Ca^{2+}$ influx by MeHg increases as a function of time of exposure in Na-free medium. Under these conditions, Na^+/Ca^{2+} exchange does not contribute significantly to $^{45}Ca^{2+}$ influx; therefore, influx occurs predominantly via Ca^{2+} channels (8, 28). These results indicate that MeHg may block more Ca^{2+} channels as the time of exposure increases or alter the inactivation rate of Ca^{2+} channels.

MeHg differs from inorganic divalent Ca^{2+} channel blockers such as Cd^{2+}, Pb^{2+}, and Hg^{2+} in that it is monovalent and has increased lipophilicity imparted by the methyl group. The ability of MeHg to block $^{45}Ca^{2+}$ influx in a voltage-dependent manner implies that the monovalent charge on MeHg may be important for the interaction of this compound with Ca^{2+} channels. The voltage dependence of block by MeHg could be related to its positive charge. Alternatively, the voltage dependence could be related to the activation of $^{45}Ca^{2+}$ influx itself, if MeHg required that Ca^{2+} channels be open to exert its blocking action. However, there is no threshold for the action of MeHg, inasmuch as block increases steadily with increasing depolarization. This is consistent with the results of state-dependence experiments discussed below. If block of Ca^{2+} channels by MeHg were state dependent, one might expect to observe a

threshold in the block of $^{45}Ca^{2+}$ influx at $[K^+]$ in the range of the activation and/or inactivation thresholds of $^{45}Ca^{2+}$ uptake. Finally, it should be noted that the voltage dependence of block could be due to nonspecific interactions of MeHg with the membrane. Sulfhydryl-reactive agents such as mercuric chloride and *para*-hydroxymercuribenzoate can depolarize the membrane (50). Thus, MeHg may also alter membrane potential. In squid axon membranes, concentrations of MeHg less than 100 μM do not cause depolarization (51) and in neuroblastoma cells 40 μM MeHg results in only 4 mV of depolarization after 6 min of exposure (52). Thus, MeHg does not cause significant depolarization of the membrane in these preparations. However, in guinea pig synaptosomes, 100 μM MeHg caused a depolarization of the membrane of 36 mV after 1 min of exposure (53). In the experiments reported in the present study, exposure to MeHg was extremely short (2 sec). Therefore, although it is possible that MeHg causes some membrane depolarization, it is unlikely that the magnitude of depolarization would be great enough to affect the results significantly. However, if MeHg were to cause significant depolarization, it might also give rise to a "voltage dependence," because it may antagonize the block by MeHg at lower K^+ concentrations by causing depolarization and opening more Ca^{2+} channels than in the control synaptosomes. We are planning further study of the voltage-dependent actions of MeHg on Ca^{2+} channels using electrophysiological techniques.

Access to the resting or open and inactivated states of synaptosomal Ca^{2+} channels did not affect the potency or efficacy of block of $^{45}Ca^{2+}$ influx by MeHg. Different association and dissociation rates for the resting, open, and inactivated states of ionic channels are thought to underlie the state-dependent block of I_{Na} by local anesthetics (35, 54, 55) and the action of certain Ca^{2+} channel blockers (56). Our results suggest that MeHg does not have an increased affinity for any particular state of the Ca^{2+} channel, because its blocking effects were equivalent following access to the channel in the presence and absence of predepolarization. This is distinct from the blocking action of DHPs, which block I_{Ca} in a state-dependent manner in cardiac myocytes (57), and the possible state dependence of Ni^{2+}, La^{3+}, and verapamil in synaptosomes (46). It should be noted that synaptosomal preparations may contain multiple types of Ca^{2+} channels, which may have different rates of inactivation. Thus, it is possible that our experiments have examined effects of MeHg on two or more subpopulations of Ca^{2+} channels and that MeHg has equal affinity for each subpopulation. However, apparent inactivation in our synaptosomal preparation was best described by a single-exponential process, which is consistent with previous results (28, 45). Therefore, we have interpreted the results of the state-dependent experiments as though the synaptosomal Ca^{2+} channel population responded to depolarization in a homogeneous manner.

Predepolarization of synaptosomes in the presence of MeHg significantly increased the rate of the apparent inactivation of $^{45}Ca^{2+}$ influx. Inactivation of synaptosomal Ca^{2+} channels with time results in decreased $^{45}Ca^{2+}$ influx; MeHg may decrease influx further by blocking open/active Ca^{2+} channels, giving rise to an increase in the rate of apparent inactivation. Intuitively, one might expect that the rate constants for normal inactivation and MeHg-induced block of Ca^{2+} channels would differ, resulting in two exponential components of inactivation, unlike the case observed in the present study. Nevertheless, the results of the present experiment cannot rule out this hypothesis definitively. Alternatively, these results may indicate an ability of MeHg to hasten the process of Ca^{2+} channel inactivation. MeHg may act directly on the Ca^{2+} channel to increase the rate of apparent inactivation or may act indirectly to increase inactivation of Ca^{2+} channels by causing release of Ca^{2+} from intracellular stores (53), resulting in increased $[Ca^{2+}]_i$, and Ca^{2+}-dependent inactivation of Ca^{2+} channels. This latter mechanism, although possible, is unlikely to contribute greatly to the increased rate of apparent inactivation of $^{45}Ca^{2+}$ influx in this experiment. It requires that MeHg diffuse through the synaptosomal membrane and interact with the mitochondria to cause Ca^{2+} release. Ca^{2+} must then diffuse back to the membrane and interact with Ca^{2+} channels to cause inactivation. This sequence of events would have to take place extremely rapidly, because apparent inactivation in the presence of MeHg occurs in less than 1 sec. In addition, the synaptosomes have been prepared, incubated, and predepolarized in nominally Ca^{2+}-free buffers. A redistribution of Ca^{2+} from the mitochondria would be expected, lowering the overall mitochondrial Ca^{2+} content.

In addition to increasing the rate of apparent inactivation, MeHg also decreases the fraction of $^{45}Ca^{2+}$ influx that does not inactivate during the predepolarization period. Noninactivating $^{45}Ca^{2+}$ influx in synaptosomes is composed of both a channel-mediated component and a reversed Na^+/Ca^{2+} exchange component (8, 28, 58). Nerve terminal Na^+/Ca^{2+} exchange appears to be insensitive to block by MeHg (8, 43); therefore, if MeHg caused significant Ca^{2+} release from mitochondria, it could decrease this phase of uptake by decreasing the driving force for exchange or by reversing exchange. Alternatively, the effect of MeHg on the noninactivating portion of Ca^{2+} influx suggests an action of MeHg on a noninactivating population of Ca^{2+} channels. This decrease may be the result of either a direct block of these channels or increased Ca^{2+}-dependent inactivation due to MeHg-induced Ca^{2+} release from intracellular stores.

MeHg also altered the relative influx of $^{45}Ca^{2+}$, $^{85}Sr^{2+}$, and $^{133}Ba^{2+}$ into synaptosomes via Ca^{2+} channels. $^{85}Sr^{2+}$ influx through Ca^{2+} channels was more sensitive to block by MeHg than was influx of $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$. This represents a unique action by MeHg on Ca^{2+} channels when compared with other divalent cations such as Ni^{2+}, which will block the influx of Ca^{2+}, Sr^{2+}, and Ba^{2+} without altering the relative influx of these three cations (40). Although these experiments do not define the site of action of MeHg, they suggest that MeHg may affect the function of the selectivity filter of the Ca^{2+} channel.

The ability of nifedipine to block the influx of $^{45}Ca^{2+}$ into PC12 cells is consistent with the action of other DHP antagonists on Ca^{2+} influx in this cell line (16, 29, 49) and indicates that depolarization-induced $^{45}Ca^{2+}$ influx into these cells is mediated largely by DHP-sensitive or L-type Ca^{2+} channels. In addition, in the present study, approximately 10% of $^{45}Ca^{2+}$ influx in differentiated PC12 cells remained after exposure to 100 nM nifedipine, indicating a DHP-insensitive portion of $^{45}Ca^{2+}$ influx. Although it would require electrophysiological experiments to confirm, the remaining influx may represent influx via an N-type channel or some as yet undescribed conductance in PC12 cells. MeHg blocked $^{45}Ca^{2+}$ influx into both nondifferentiated and NGF-differentiated PC12 cells at concentrations similar to those that block $^{45}Ca^{2+}$ influx into synaptosomes (8). In two of three experiments, MeHg completely

blocked $^{45}Ca^{2+}$ influx into NGF-differentiated PC12 cells. This indicates that MeHg may be capable of blocking a DHP-insensitive Ca^{2+} channel as well as a DHP-sensitive channel. The ability of MeHg to block DHP-sensitive or L-type Ca^{2+} channels in PC12 cells is important in light of recent studies that indicate that these channels may be responsible for, or at least modulate, Ca^{2+} entry associated with release of some neurotransmitters from neurons (15, 59–62) and PC12 cells (23, 49). In addition, DHP-insensitive Ca^{2+} conductances have been reported to mediate, in part, release of [^{3}H]norepinephrine from NGF-differentiated PC12 cells (49). Thus, MeHg is capable of blocking at least one and possibly two types of Ca^{2+} channels that are associated with neurotransmitter release. This action may contribute in large measure to the observation that MeHg depresses Ca^{2+}-dependent transmitter release (5).

High-affinity binding of DHPs has been demonstrated in synaptosomes (27, 28, 63, 64). However, the relationship between binding of DHPs and effects of DHPs on $^{45}Ca^{2+}$ influx into synaptosomes is unclear. Several studies (28, 30, 45, 65–67) indicate that Ca^{2+} influx into synaptosomes is insensitive to block by DHP antagonists, whereas other studies (27, 64, 68) indicate that a portion of Ca^{2+} influx in synaptosomes is sensitive to DHP antagonists. In either case, the concentrations of DHP required to block $^{45}Ca^{2+}$ influx are in great excess of those at which DHPs bind specifically to synaptosomes. In the present study, [^{3}H]nitrendipine bound to a single class of high affinity sites in rat forebrain synaptosomes. The values for K_D and B_{max} correlate well with values observed by others for nitrendipine binding in synaptosomes (27, 28). DHP agonists and antagonists are thought to bind to Ca^{2+} channels more readily when the channel is in the open or inactivated state, respectively (57). However, depolarization of the synaptosomes did not readily affect the affinity of nitrendipine binding or the maximum number of binding sites. The binding affinity of the DHP antagonist (+)-PN200–110 to synaptosomes was decreased by K^+-induced depolarization (64). Although other explanations are possible, the lack of effect of depolarization on the affinity of DHP binding would be consistent with the suggestion that DHP binding sites in synaptosomal preparations are associated with fragments of postsynaptic membrane that remain attached to the synaptosomes (45). These fragments could not maintain a membrane potential and, hence, would not respond to changes in K^+ concentration. The affinity of nitrendipine binding to synaptosomes was decreased in the presence of a concentration of MeHg that readily blocks $^{45}Ca^{2+}$ influx in synaptosomes (8) and DHP-sensitive influx in PC12 cells. The ability of MeHg to compete with nitrendipine for a high-affinity binding site in synaptosomes is consistent with its ability to block DHP-sensitive channels in PC12 cells. To the best of our knowledge, binding of [^{3}H]nitrendipine to intact PC12 cells has not been demonstrated, and we have not been able to demonstrate appreciable specific binding of nitrendipine to intact PC12 cells.

Caution must be observed in correlating the results of binding studies in synaptosomes with flux studies in PC12 cells and concluding that MeHg blocks a DHP-sensitive Ca^{2+} channel in synaptosomes. According to one model for the movement of Ca^{2+} through DHP-sensitive cardiac Ca^{2+} channels, higher concentrations of Ca^{2+} are necessary to observe a Ca^{2+} current (69) than are normally employed in our experiments with synaptosomes. Thus, the fluxes that we commonly measure may not necessarily be mediated by DHP-sensitive Ca^{2+} channels. As shown in Fig. 5, MeHg will block $^{45}Ca^{2+}$ influx into synaptosomes at concentrations of Ca^{2+} high enough to mediate flux via L-type channels. However, we have not determined the contribution, if any, of $^{45}Ca^{2+}$ flux into synaptosomes mediated by L-type channels under these conditions.

The polypeptide CgTx binds to Ca^{2+} channels at a site that is distinct from the DHP binding site (31–34) and in some axon terminals is thought to be associated with transmitter release. As opposed to nitrendipine, MeHg did not alter the binding of CgTx to synaptosomes. This indicates that MeHg does not interact with this site in synaptosomes or that CgTx displaces MeHg from this site. The effects of MeHg on $^{45}Ca^{2+}$ uptake in synaptosomes are only partially reversible by increasing $[Ca^{2+}]_e$ (8) and at neuromuscular junctions are only reversible under certain conditions (5). Thus, it seemed likely that MeHg is tightly or irreversibly bound to receptors on the cell surface. Consequently, experiments were designed to test whether pretreatment with MeHg might alter CgTx binding to its receptor in synaptosomes. Our results suggest that MeHg does not interact with the CgTx binding site in synaptosomes, because CgTx binding was not altered by pretreatment with MeHg or incubation in the presence of MeHg.

In contrast to the results of synaptosomal experiments, MeHg did alter the binding of CgTx to PC12 cells. The amount of CgTx bound to PC12 cells was decreased significantly in the presence of MeHg at concentrations that effectively block $^{45}Ca^{2+}$ entry into this cell line. Thus, MeHg apparently interacts with the CgTx receptor in PC12 cells but not on synaptosomes. This suggests that the CgTx receptor, and hence perhaps the Ca^{2+} channels, in synaptosomes and PC12 cells are different. CgTx is thought to interact with N-type and possibly L-type channels in mammalian neurons (67). In both synaptosomes and PC12 cells, our results indicate the presence of only a single class of CgTx receptor; thus, it is possible that CgTx binds to one channel type in synaptosomes and the other type in PC12 cells. However, it is difficult to interpret the results of CgTx binding experiments, because the type(s) of Ca^{2+} channel(s) in synaptosomes are unknown and, in PC12 cells, only N-type conductances are sensitive to block by CgTx (23). Although the latter indicates that CgTx may not block L-type channels in PC12 cells, it does not preclude binding to this channel type from taking place. Thus, it is unclear to which Ca^{2+} channel subtype CgTx is binding in our preparations. Alternatively, CgTx may be binding to a site in our synaptosomes and/or PC12 cells that is not associated with Ca^{2+} channels. Much higher concentrations of CgTx are required to block $^{45}Ca^{2+}$ influx in mammalian synaptosomes than in avian or amphibian synaptosomes (70), in spite of the presence of high-affinity binding sites for CgTx. Thus, interpretation of these data beyond the conclusion that MeHg interacts with the CgTx receptor in PC12 cells, but not synaptosomes, would be purely speculative.

MeHg is a very reactive heavy metal and alters a number of neuronal functions. Although effects of MeHg on Ca^{2+} channels are probably not solely responsible for the neurotoxic actions of MeHg, the rapid action of MeHg on Ca^{2+} influx into synaptosomes indicates that nerve terminal Ca^{2+} channels may be an initial site of interaction of MeHg with neurons. In summary, the results of this study suggest that MeHg interacts with nerve terminal Ca^{2+} channels in a manner unique from that of inorganic divalent cations or DHPs. MeHg clearly interacts with a DHP-sensitive Ca^{2+} channel in PC12 cells, but

the relationship of this interaction to the effects of MeHg on $^{45}Ca^{2+}$ influx in synaptosomes and synaptic transmission at the neuromuscular junction is unclear. In addition, MeHg does not block Ca^{2+} channels in a state-dependent manner, as do DHPs. MeHg is also unique from other neurotoxic heavy metals in its ability to alter both the apparent inactivation kinetics and ionic selectivity of fluxes through the Ca^{2+} channel. Differences in lipophilicity and charge may underlie the unique blocking action of MeHg on Ca^{2+} channels.

Acknowledgments

The authors acknowledge the generous gift of NGF from Dr. Steven Heidemann, Department of Physiology, Michigan State University, and thank Dr. Gregory Fink, Department of Pharmacology and Toxicology, Michigan State University, for his assistance with statistical analyses. The excellent technical assistance of Lynne Adgate, Beth Bigler, Wendy Pittman, Jacqueline Stout, Dawn Terrasas, and Jennise Vincent and the excellent secretarial assistance of Diane Hummel are appreciated.

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