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Loperamide Blocks High-Voltage-Activated Calcium Channels and *N*-Methyl-D-Aspartate-Evoked Responses in Rat and Mouse Cultured Hippocampal Pyramidal Neurons

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SUMMARY

The effects of the antidiarrheal agent loperamide on high-voltage-activated (HVA) calcium channel activity and excitatory amino acid-evoked responses in two preparations of cultured hippocampal pyramidal neurons were examined. In rat hippocampal neurons loaded with the calcium-sensitive dye fura-2, rises in intracellular free calcium concentration ($[Ca^{2+}]_i$) evoked by transient exposure to 50 mM K^+-containing medium [high extracellular potassium concentration ($[K^+]_o$)] were mediated by Ca^{2+} flux largely through nifedipine-sensitive Ca^{2+} channels, with smaller contributions from ω-conotoxin GVIA (ω-CgTx)-sensitive Ca^{2+} channels and channels insensitive to both nifedipine and ω-CgTx. Loperamide reversibly blocked rises in $[Ca^{2+}]_i$ evoked by high $[K^+]_o$ in a concentration-dependent manner, with an IC_{50} of 0.9 ± 0.2 μM. At the highest concentration tested (50 μM), loperamide eliminated rises in $[Ca^{2+}]_i$ evoked by high $[K^+]_o$, a result otherwise achieved only in Ca^{2+}-free medium or by the combined application of nifedipine, ω-CgTx, and funnel web spider venom to Ca^{2+}-containing medium. The action of loperamide was neither naloxone sensitive nor mimicked by morphine and was seen at concentrations substantially less than those required to block influx of Ca^{2+} through the *N*-methyl-D-aspartate (NMDA) receptor-operated ionophore. Similar results were obtained in cultured mouse hippocampal pyramidal neurons under whole-cell voltage clamp. Voltage-activated Ca^{2+} channel currents carried by barium ions (I_{Ba}) could be discriminated pharmacologically into nifedipine-sensitive (L-type) and nifedipine-resistant, ω-CgTx-sensitive (N-type) components. Loperamide (0.1–50 μM) produced a concentration-dependent reduction of the peak I_{Ba} with an IC_{50} value of 2.5 ± 0.4 μM and, at the highest concentration tested, could fully block I_{Ba} in the absence of any other pharmacological agent. The loperamide-induced block was rapid in onset and offset, was fully reversible, and did not appear to be related to the known calmodulin antagonist actions of loperamide. The current-voltage characteristics of the whole-cell I_{Ba} were unaffected by loperamide and the block was not voltage dependent. Loperamide also attenuated NMDA-evoked currents recorded at a membrane potential of −60 mV, with an IC_{50} of 73 ± 7 μM. The block of NMDA-evoked currents was not competitive in nature, was not reversed by elevation of the extracellular glycine or spermine concentration, and was not affected by changes in the membrane holding potential. Steady state currents evoked by kainate and DL-α-amino-3-hydroxy-5-methylisoxazolepropionic acid were, in contrast, relatively unaffected by 100 μM loperamide. We conclude that loperamide, applied at low micromolar concentrations, is a broad-spectrum blocker of neuronal HVA Ca^{2+} channels. At higher concentrations, it reduces Ca^{2+} flux through NMDA receptor-operated channels. Loperamide may prove to be a useful tool in experiments in which a general and reversible suppression of neuronal HVA Ca^{2+} channel activity is required.

Loperamide is widely used as an antidiarrheal agent (reviewed in Ref. 1). Chemically a hybrid of the narcotic analgesic meperidine and the neuroleptic haloperidol, it inhibits gastrointestinal motility in a morphine-like, naloxone-sensitive manner and, indeed, it has high affinity for both peripheral and central opioid receptors (2). In addition, however, loperamide exhibits a variety of non-opioid receptor-mediated effects, including a Ca^{2+} channel-blocking action. For example, loperamide blocks Ca^{2+}-induced contractions of ileal smooth muscle (3, 4) and depresses acetylcholine release, not only from colonic but also from bronchial cholinergic nerve terminals (5, 6). Interestingly, the ability of loperamide to attenuate Ca^{2+}-induced ileal contractions is shared by the antitussive DXM (4), which, in addition to its established NMDA antagonist actions, possesses

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ABBREVIATIONS: DXM, dextromethorphan; NMDA, *N*-methyl-D-aspartic acid; HVA, high-voltage-activated; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AMPA, DL-α-amino-3-hydroxy-5-methylisoxazolepropionic acid; ω-CgTx, ω-conotoxin GVIA; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; AP5, (±)-2-amino-5-phosphonopentanoic acid; I_{Ba}, barium current; $[Ca^{2+}]_i$, intracellular free calcium concentration; $[K^+]_o$, extracellular potassium concentration.

affinity for [^{3}H]dihydropyridine binding sites and blocks Ca^{2+} flux through neuronal voltage-activated Ca^{2+} channels (7–10). Loperamide also has high affinity for [^{3}H]dihydropyridine binding sites (3), although cross-displacement experiments between DXM and loperamide have not been reported.

In the present study, we have therefore examined the selectivity and potency of loperamide as a blocker of HVA Ca^{2+} channels and as an excitatory amino acid antagonist in cultured hippocampal pyramidal neurons, either loaded with the Ca^{2+}-sensitive dye fura-2 or under whole-cell voltage clamp. We find that loperamide at low micromolar concentrations is a broad-spectrum blocker of neuronal HVA Ca^{2+} channels, whereas at higher concentrations it produces a reduction of NMDA-evoked responses.

Materials and Methods

Fluorescent Dye Studies

Hippocampal pyramidal neurons obtained from 18-day-old fetal Wistar rats (11) were plated on glass coverslips at a density of 1–3 × 10^5 cells/cm^2 and were used 6–16 days after plating. After loading with fura-2 (see below), coverslips were placed in a chamber at 20–23° and continuously superfused at a rate of 1.5 ml/min with a solution containing 136.5 mM NaCl, 3 mM KCl, 1.5 mM NaH_2PO_4, 1.5 mM $MgSO_4$, 10 mM D-glucose, 2 mM $CaCl_2$, and 10 mM HEPES. Tetrodotoxin (0.5 μM) was added and the pH was adjusted to 7.35–7.40 with 10 M NaOH. In experiments using NMDA, Mg^{2+} was omitted and 2 μM glycine was added. For Ca^{2+}-free solutions, Ca^{2+} was omitted, the Mg^{2+} concentration was increased to 4 mM, and 100 μM EGTA was added. The amino acid excitants NMDA (20 μM), kainate (80 μM), and AMPA (40 μM) were administered in 1-ml aliquots to the inflow of the perfusion chamber and were allowed to remain in contact with the neurons for 20 sec before wash-out. High-$[K^+]_o$ solutions (50 mM, by substitution for NaCl) were introduced in a similar fashion. Test compounds were applied by superfusion; their effects were expressed as the percentage change of the peak response to an excitant during their administration, in relation to the peaks of the control and, where reversible effects occurred, the recovery responses obtained before and after their administration.

$[Ca^{2+}]_i$ values were measured by the dual-excitation fluorescence ratio method, with an Attofluor digital fluorescence microscopy system (Atto Instruments Inc.; Carl Zeiss Canada Ltd.), using the Ca^{2+}-sensitive fluorophore fura-2 (12). Neurons were incubated at 33–35° for 60–70 min with 7.5 μM fura-2/acetoxymethyl ester (Molecular Probes Inc.) and were then washed and left for 30 min before use, to ensure complete hydrolysis of the acetoxymethyl ester form of fura-2. Using excitation wavelengths of 334 and 380 nm, fluorescence intensities (at 510 nm) were obtained from multiple neuron somata simultaneously. Raw intensity data at each excitation wavelength were corrected for background levels before calculation of the ratio. During exposure to excitants, a ratio was acquired every 1.5 sec; lower rates (e.g., one ratio every 30 sec) were used between excitant applications to minimize photobleaching, UV-mediated cytotoxicity, and, in the case of nifedipine-containing solutions, drug breakdown. The *in situ* calibration method was used to convert fluorescence ratios into $[Ca^{2+}]_i$ values. During exposure to 10 μM Br-A23187, calibration parameters (R_{max}, R_{min}, and β) were obtained in the presence (2 mM) and absence (nominally Ca^{2+}-free solution in the presence of 100 μM EGTA) of Ca^{2+}. The published K_d value of 135 nM (at 20°) was used (12).

Statistical results are reported as mean ± standard error, where n refers to the total number of neurons from which observations were made under each experimental condition. Each experiment was performed on at least three different neuronal cultures. To derive IC_{50} values (the concentration of test compound resulting in 50% inhibition of the control response), averaged data points were fitted to the logistic equation $R = R_{max}[\text{concentration}^n/(\text{concentration}^n + IC_{50}^n)]$, where R is the observed change at the test concentration, R_{max} is the maximum observed change, concentration refers to the test concentration, and n_H is the Hill coefficient.

Electrophysiological Studies

Dissociated mouse hippocampal pyramidal neurons grown in culture were used for whole-cell recordings using conventional voltage-clamp techniques. The method of culture preparation has been described previously (13). In brief, hippocampi were dissected from 18-day-old fetal Swiss White mice and mechanically dissociated, and neurons were plated at densities below 1 × 10^3 cells/cm^2, on collagen-coated plates. Cultures were used 7–14 days after plating. Recordings were performed at room temperature (20–24°).

Amino acid-evoked responses. For studies of amino acid-evoked responses, culture plates were initially rinsed before the commencement of the experiment with an extracellular solution containing 140 mM NaCl, 1.3 mM $CaCl_2$, 5.4 mM KCl, 25 mM HEPES, and 33 mM glucose (final osmolarity, 330 mOsM). Tetrodotoxin (100 nM) and glycine (3 μM) (or as indicated) were added before the pH of the solution was adjusted to 7.35–7.40 with 1 M NaOH. Agonists and antagonists were applied to neurons from a three-barreled perfusion system allowing for rapid (<50-msec) application of compounds to the entire neuron under voltage clamp (14). Patch electrodes were filled with a solution containing 110 mM CsF, 10 mM CsCl, 10 mM HEPES, and 10 mM EGTA, pH 7.35–7.40 (adjusted with 2 M CsOH). Occasionally, an intracellular solution containing 125 mM cesium methanesulfonate, 15 mM CsCl, 10 mM HEPES, 5 mM EGTA, 0.5 mM $CaCl_2$, and 1 mM $MgCl_2$ was used. The membrane of the neuron was maintained at −60 mV, unless otherwise indicated, using a patch-clamp amplifier (Axopatch 1B; Axon Instruments Inc.).

Responses evoked upon amino acid application were recorded using pClamp acquisition and analysis software (Axon Instruments Inc.). In general, three responses were averaged for each data point and drug effect was expressed as the percentage reduction of the control agonist response at steady state. The final IC_{50} values and percentage reductions of control responses are expressed as mean ± standard error, with n being the number of neurons tested. EC_{50} values (the concentration of NMDA producing a response 50% of the maximal amplitude) and IC_{50} values were estimated by fitting data points to the same logistic equation as in the fluorescent dye experiments (see above).

I_{Ba} measurements. For studies of voltage-activated Ca^{2+} channels, the extracellular solution (see above) was changed, after the whole-cell voltage-clamp configuration was attained, to one containing 140 mM NaCl, 5 mM CsCl, 5 mM $BaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, and glucose to a final osmolarity of 330 mOsM. To achieve the best possible space clamp of the membrane, a higher concentration of tetrodotoxin (300–600 nM) was added to block voltage-activated Na^+ currents; K^+ currents were minimized by replacing extracellular KCl with CsCl. The series resistance and capacitance of the electrode were compensated for by using the patch-clamp amplifier, although compensation for those of the neuronal membrane could only partially be achieved in the cultured cell preparation used. In all cases leak currents were estimated by superfusion with 100 μM cadmium-containing solution. A fluoride-free intracellular solution containing 110 mM CsCl, 10 mM tetraethylammonium, 10 mM EGTA, and 10 mM HEPES was used. On the day of the experiment, a support system was added that included 2 mM Mg-ATP, 50 mM phosphocreatine, 50 IU of creatine phosphokinase (13), and 2 mM GTP. The pH and osmolarity of the solution were then adjusted to 7.35–7.40 and 310 mOsM, respectively. The final solution was kept on ice for the duration of the experiment and was rarely used if >3 hr old, due to instability of the components of the support system.

Voltage-operated Ca^{2+} channels were activated at 20-sec intervals by a 250-msec voltage step to a test potential of −10 mV from a holding potential of −80 mV. Other protocols used are detailed in the Results section, under Electrophysiological Studies. The aforementioned step in potential has been established to activate both nifedipine-sensitive (L-type) and nifedipine-resistant, ω-CgTx-sensitive (N-type) voltage-

activated Ca^{2+} channels in hippocampal neurons (e.g., see Refs. 15 and 16). Small currents mediated by low-voltage-activated (T-type) Ca^{2+} channels were seen infrequently in the cultures used, as reported elsewhere (15–18), and we did not attempt to study the effects of loperamide on this Ca^{2+} channel subtype. Loperamide was perfused over the neuron and I_{Ba} was activated until steady state block was achieved. For construction of concentration-inhibition plots (as described above), the concentration of loperamide was sequentially increased to achieve full block before the start of wash-out, to minimize the possible error contributed by run-down of I_{Ba}. Cells that displayed a <50% recovery of control I_{Ba} were excluded from analysis.

Sources and Handling of Compounds

Compounds were obtained from Sigma Chemical Co., with the exceptions of AMPA, CNQX, AP5, naloxone, ω-CgTx (Research Biochemicals Inc.), and funnel web spider (*Agelenopsis aperta*) venom (Spider Pharm Inc., Black Canyon City, AZ). ω-CgTx (250 μM stock solution in distilled, O_2-free water) and funnel web spider venom were stored at −60°. Stock solutions of loperamide (50 mM in dimethylsulfoxide) and nifedipine (10 or 50 mM in ethanol) were made up fresh each day from the solid compounds and were stored in glass vials. The highest concentration of either dimethylsulfoxide or ethanol in the final working solutions was 0.1%, which in control experiments had no effect on responses (data not shown). Nifedipine-containing solutions were handled in the manner described by McCarthy and TanPiengco (19) for nimodipine.

Results

Fluorescent Dye Studies

Characterization of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. Transient exposure to 50 mM K^+ produced a rise in $[Ca^{2+}]_i$ of 1161 ± 40 nM (n = 1117). During perfusion with Ca^{2+}-free medium, high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ were reduced by 98 ± 1% (n = 70), indicating the dependence of the response on extracellular Ca^{2+} (data not shown). Addition of 40 μM AP5 and 20 μM CNQX to the perfusion medium reduced the magnitude of the rise in $[Ca^{2+}]_i$ evoked by high $[K^+]_o$ by 5 ± 2% (n = 109), suggesting that endogenously released glutamate contributed little to the observed response (see Fig. 1b).

Selective blockers of neuronal HVA Ca^{2+} channels (reviewed in Refs. 20–23) were used to investigate the contribution made by each subtype of Ca^{2+} channel to the overall high-$[K^+]_o$-evoked response. Nifedipine, a blocker of L-type calcium channels, produced a concentration-dependent reduction of the high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$, with an apparent IC_{50} of 11 ± 2 nM (Figs. 1 and 2). Nifedipine alone was unable to block completely the high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$; a maximal reduction of approximately 80–85% was seen at a concentration of 1–2 μM, with no additional block with further increases in nifedipine concentration (Figs. 1a and 2b) (15, 24, 25). Under experimental conditions similar to our own, Thayer *et al.* (26) reported a 79% reduction by 1 μM nitrendipine of 50 mM K^+-evoked rises in $[Ca^{2+}]_i$ in cultured rat hippocampal cells. At concentrations of ≥5 μM, the effect of nifedipine was only slowly reversible (Fig. 1a) (15, 24).

High-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ that were resistant to 10 μM nifedipine were sensitive to ω-CgTx and funnel web spider venom (Fig. 1b) (16, 25, 27–31). Examined in a total of 109 neurons, high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ were reduced by 82 ± 2% in the presence of 40 μM AP5, 20 μM CNQX, and 10 μM nifedipine. Addition of 10 μM ω-CgTx produced a 49 ± 2% reduction of the AP5-, CNQX-, and nifedipine-resistant residual response, resulting in an overall reduction of the control, drug-free, high-$[K^+]_o$-evoked response of 91%. Under similar experimental conditions, Ogura *et al.* (32) reported a 92% reduction of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ during exposure to 10 μM nitrendipine and 0.1 μM ω-CgTx. In turn, the residual high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$ seen in the presence of AP5, CNQX, nifedipine, and ω-CgTx was irreversibly blocked by the addition of funnel web spider venom (1/1000 dilution) (33).

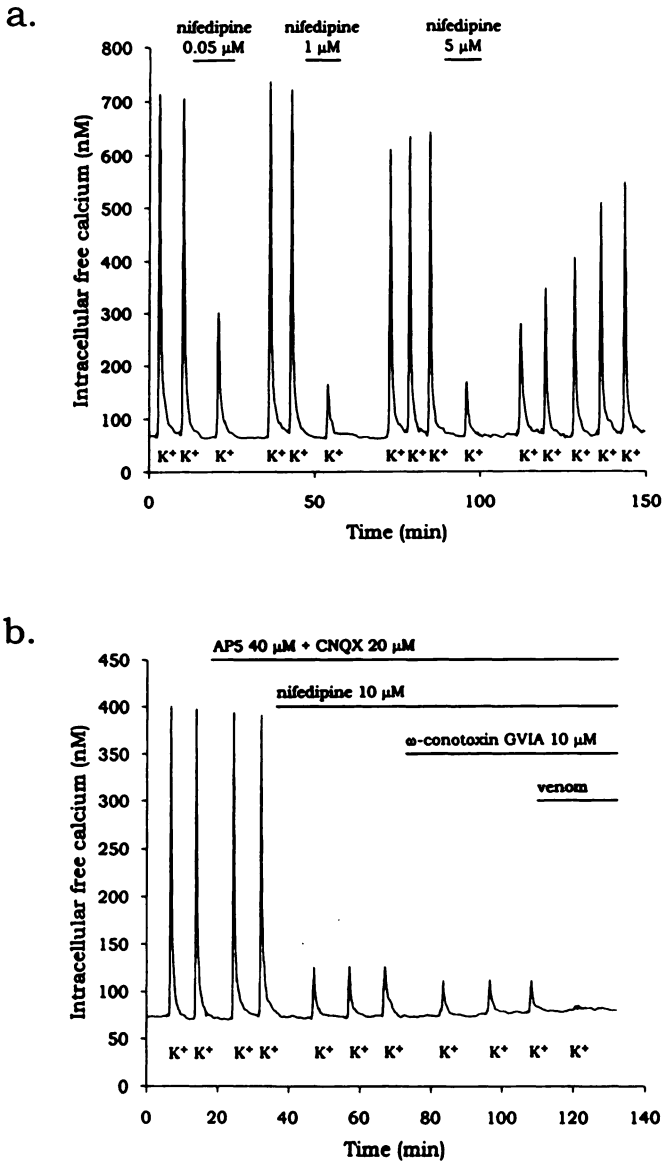

Fig. 1. Pharmacological characterization of high-$[K^+]_o$-evoked increases in $[Ca^{2+}]_i$. a, Nifedipine (0.05–5 μM), applied for the periods indicated by the *bars above the trace*, reduced high-$[K^+]_o$ (K^+)-evoked rises in $[Ca^{2+}]_i$. Increasing the concentration of nifedipine from 1 to 5 μM failed to increase the magnitude of the block. Note also the slow recovery after exposure to 5 μM nifedipine. The record is a mean of data obtained from 12 neurons simultaneously. b, Under control conditions two consecutive exposures to 50 mM K^+ (K^+) evoked stable rises in $[Ca^{2+}]_i$. Addition of AP5 and CNQX to the perfusion medium (third and fourth responses) failed to affect the high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. Subsequent addition of 10 μM nifedipine (fifth, sixth, and seventh responses) produced a stable 85% reduction of the rise in $[Ca^{2+}]_i$ from responses obtained in the presence of AP5 and CNQX. The nifedipine-resistant residual was then reduced by 37% by addition of 10 μM ω-CgTx (eighth, ninth, and 10th responses). Finally, addition of funnel web spider venom (1/1000 dilution; 11th response) reduced the nifedipine- and ω-CgTx-resistant residual by >90%. The record shows the mean response of 14 neurons.

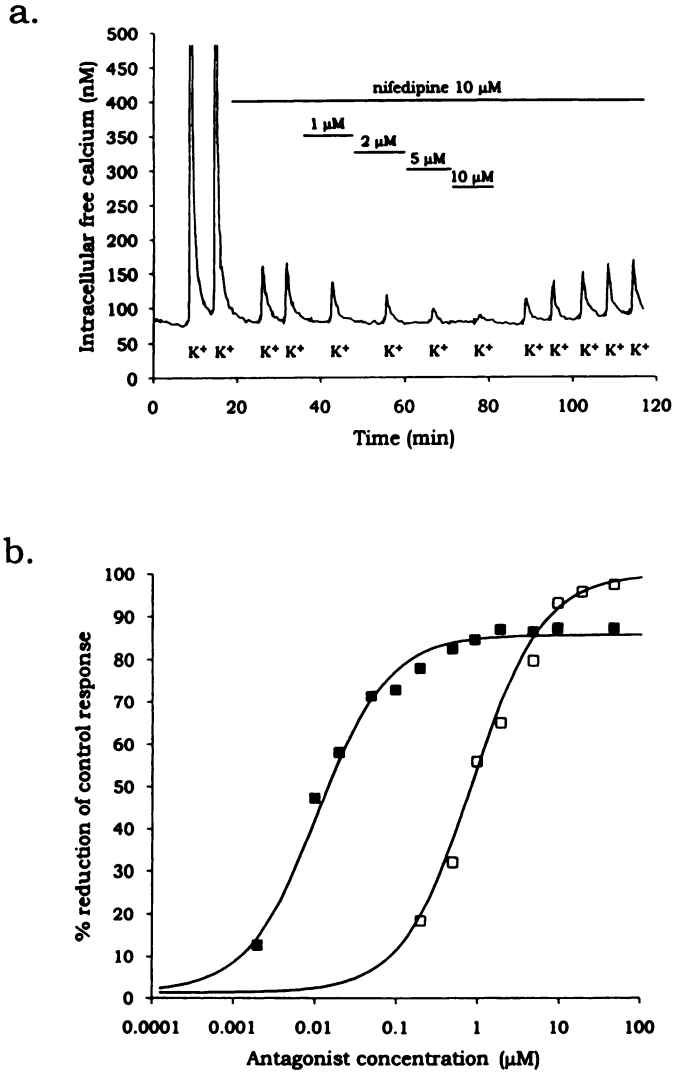

Fig. 2. a, Loperamide produces a concentration-dependent reduction of nifedipine-resistant high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. Under control conditions (first and second responses; peaks truncated for convenience in figure preparation), 50 mM K^+ (K^+) evoked large rises in $[Ca^{2+}]_i$, which were then reduced by 87% by 10 μM nifedipine (third and fourth responses). Coapplication of 1–10 μM loperamide, for the periods indicated by the *bars above the trace*, produced a concentration-dependent and fully reversible reduction in the nifedipine-resistant residual response. The experiment was conducted in the presence of 40 μM AP5 and 20 μM CNQX. The record is a mean of data obtained from 17 neurons simultaneously. b, Concentration-inhibition plots for nifedipine (■) and loperamide (□) as blockers of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. Data points indicate means and were fitted using a logistic equation (see Materials and Methods); standard error bars lie within the symbol areas. IC_{50} values for nifedipine and loperamide against high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ were 0.011 ± 0.002 and 0.87 ± 0.24 μM, respectively.

The results indicate that, under our experimental conditions, transient exposure to 50 mM K^+-containing medium evokes a rise in $[Ca^{2+}]_i$ mediated largely by Ca^{2+} flux through dihydropyridine-sensitive (presumed L-type) HVA Ca^{2+} channels, with a smaller contribution from dihydropyridine-resistant, ω-CgTx-sensitive (presumed N-type), HVA Ca^{2+} channels. The small component of the high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$ that is resistant to nifedipine and ω-CgTx may represent Ca^{2+} flux through P-type HVA Ca^{2+} channels (16, 30, 31), although additional experiments with purified funnel web spider venom fractions are required to substantiate this possibility. It is unlikely that Ca^{2+} flux through low-voltage-activated (T-type) Ca^{2+} channels contributes to the high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$, given the time course of the K^+-evoked response together with the small conductance and relatively rapid inactivation of T-type Ca^{2+} channels (see Ref. 22).

Effect of loperamide on high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. Loperamide (0.1–50 μM) produced a concentration-dependent, reversible reduction of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$, with an IC_{50} of 0.87 ± 0.24 μM ($n > 60$ at each concentration tested) (Figs. 2b, 3, and 4). The effect was not accompanied by changes in resting $[Ca^{2+}]_i$ and was fully developed during the first high-$[K^+]_o$-evoked response after the start of loperamide application (data not shown). In contrast to nifedipine, loperamide applied alone at concentrations of >20 μM was able to eliminate high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$, a result otherwise achieved only during perfusion with Ca^{2+}-free medium or after the addition of nifedipine, ω-CgTx, and funnel web spider venom to Ca^{2+}-containing medium. Thus, 50 μM loperamide produced a 97 ± 1% ($n = 83$) reduction of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$; in 74 of these neurons, a ≥97% reduction was observed. When applied in the presence of 40 μM AP5, 20 μM CNQX, and 10 μM nifedipine, loperamide at 0.1–20 μM ($n > 30$ at each concentration tested) produced a concentration-dependent reduction of the nifedipine-resistant, ω-CgTx-sensitive, funnel web spider venom-sensitive high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$, with an IC_{50} value of 1.46 ± 0.47 μM (Fig. 2a).

The effect of loperamide on high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ was not naloxone sensitive and was not mimicked by morphine. In the absence of naloxone, 5 μM loperamide produced an 80 ± 1% reduction of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ ($n = 126$), a result not significantly different from its effects in the presence of 5 μM naloxone (an 81 ± 1% reduction; $n = 61$; $p > 0.8$, Student's unpaired t test) (Fig. 4a). Morphine, applied at 10 and 20 μM, produced a 4 ± 4% ($n = 30$) and 1 ± 5% ($n = 23$) increase, respectively, in rises in $[Ca^{2+}]_i$ evoked by 50 mM K^+ (data not shown).

Effect of loperamide on excitatory amino acid-evoked rises in $[Ca^{2+}]_i$. Application of AMPA or kainate evoked rises in $[Ca^{2+}]_i$ of 1073 ± 136 nM ($n = 114$) and 1131 ± 145 nM ($n = 53$), respectively. As previously reported (e.g., see Ref. 34), these responses were dependent on the presence of extracellular calcium, being depressed by 96 ± 1% ($n = 26$) and 97 ± 0.2% ($n = 26$), respectively, in the absence of extracellular Ca^{2+} (data not shown). Loperamide (0.1–50 μM) reduced AMPA- and kainate-evoked responses to a similar extent as the high-$[K^+]_o$-evoked responses (Fig. 4b). Thus, for example, loperamide at 2 and 5 μM reduced AMPA-evoked rises in $[Ca^{2+}]_i$ by 62 ± 2% ($n = 27$) and 74 ± 2% ($n = 40$), respectively; corresponding reductions of high-$[K^+]_o$-evoked responses were 65 ± 1% ($n = 90$) and 79 ± 1% ($n = 126$), respectively. Similarly, loperamide at 2 and 10 μM reduced kainate-evoked rises in $[Ca^{2+}]_i$ by 64 ± 2% ($n = 27$) and 90 ± 1% ($n = 27$), respectively; the corresponding reduction of high-$[K^+]_o$-evoked responses by loperamide at 10 μM was 93 ± 0.4% ($n = 92$). Our data are entirely consistent with previous reports (34) that rises in $[Ca^{2+}]_i$ evoked by AMPA and kainate are primarily due to membrane depolarization and subsequent Ca^{2+} flux through

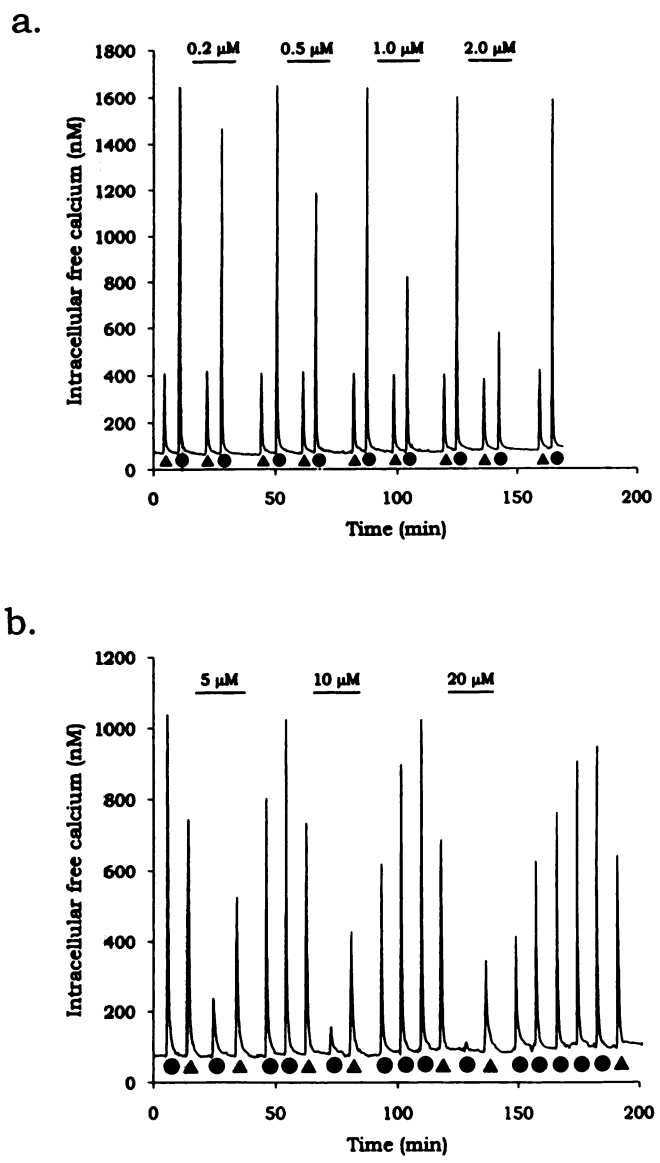

Fig. 3. Loperamide reduces high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ in a concentration-dependent manner. a, Effects of 0.2–2.0 μM loperamide, applied for the periods indicated by the *bars above the trace*, on NMDA (▲)- and high-$[K^+]_o$ (●)-evoked increases in $[Ca^{2+}]_i$. Loperamide produced a concentration-dependent reduction in high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. The record is a mean of data obtained from 14 neurons simultaneously. b, Effects of 5–20 μM loperamide, applied for the periods indicated by the *bars above the trace*, on high-$[K^+]_o$ (●)- and NMDA (▲)-evoked increases in $[Ca^{2+}]_i$. The small high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$ seen in the presence of 20 μM loperamide was subsequently abolished by 50 μM loperamide (data not shown). A concentration-dependent reduction of NMDA-evoked rises in $[Ca^{2+}]_i$ is also seen (see text). The record is a mean of data obtained from eight neurons simultaneously, in a culture different from that used in a.

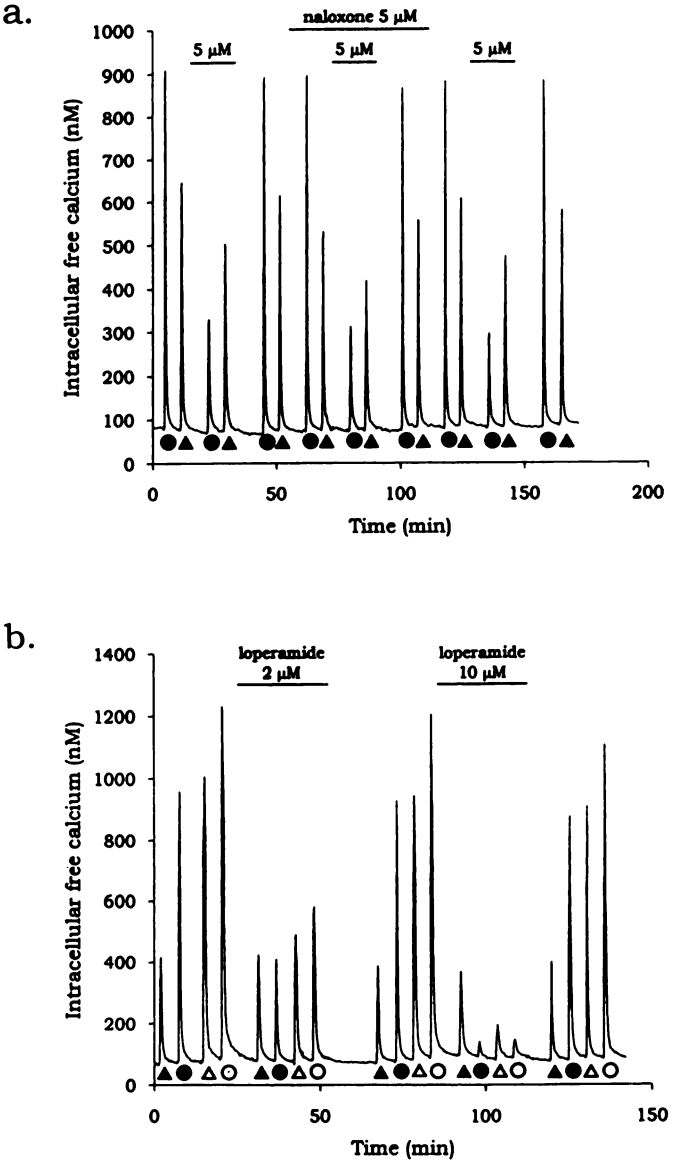

Fig. 4. a, The effect of loperamide on high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ is naloxone insensitive. Rises in $[Ca^{2+}]_i$ were evoked by high $[K^+]_o$ (●) or NMDA (▲). Loperamide (5 μM), applied for the periods indicated by the *bars above the trace*, reduced high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ to a similar extent in the presence or absence of 5 μM naloxone. The record is a mean of data obtained from 17 neurons simultaneously. b, Effect of loperamide at 2 and 10 μM, applied for the periods indicated by the *bars above the trace*, on NMDA (▲)-, high-$[K^+]_o$ (●)-, AMPA (△)-, and kainate (○)-evoked rises in $[Ca^{2+}]_i$. AMPA- and kainate-evoked responses were reduced to a similar degree as were the high-$[K^+]_o$-evoked responses. The record is a mean of data obtained from nine neurons simultaneously.

voltage-activated Ca^{2+} channels. The reduction by loperamide of AMPA- and kainate-evoked rises in $[Ca^{2+}]_i$ under our experimental conditions reflects, therefore, its blockade of HVA Ca^{2+} channels rather than non-NMDA receptor antagonist activity. The lack of activity of loperamide on AMPA- and kainate-evoked responses was confirmed in the electrophysiological studies (see below).

Application of NMDA evoked rises in $[Ca^{2+}]_i$ of 629 ± 20 nM (n = 505). Loperamide at 0.2–50 μM produced a concentration-dependent reduction in the rise in $[Ca^{2+}]_i$ evoked by NMDA (n ≥ 60 at each concentration tested) (Figs. 3 and 4b). Under our experimental conditions, a portion of this effect represents blockade by loperamide of HVA Ca^{2+} channels activated as a consequence of membrane depolarization evoked by NMDA (see Ref. 32). Nevertheless, loperamide at 50 μM produced a greater reduction of NMDA-evoked rises in $[Ca^{2+}]_i$ (60 ± 3% reduction, n = 60) than could be achieved by maximally effective concentrations (≥5 μM) of nifedipine (48 ± 3% reduction, n = 86), suggesting that loperamide, at concentrations higher than those required to substantially attenuate high-$[K^+]_o$-

evoked rises in $[Ca^{2+}]_i$, possessed some NMDA antagonist activity. This possibility was confirmed in the electrophysiological studies (see below). As in the case of high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$, naloxone had little effect on the magnitude of the reduction by loperamide of NMDA-evoked rises in $[Ca^{2+}]_i$. In the absence of naloxone, loperamide at 5 μM produced a 36 ± 2% reduction in NMDA-evoked rises in $[Ca^{2+}]_i$ (n = 126), a result not significantly different from the effect of the same concentration of the drug applied in the presence of 5 μM naloxone (a 34 ± 3% reduction; n = 41; $p > 0.4$, Student's unpaired t test).

Electrophysiological Studies

Reduction by loperamide of HVA Ca^{2+} channel currents. Loperamide at 0.1–50 μM reversibly attenuated the whole-cell I_{Ba} in the 57 neurons tested (Fig. 5). Loperamide reduced the peak amplitude of I_{Ba} in a concentration-dependent manner with an IC_{50} of 2.47 ± 0.44 μM (n = 12) and that of the delayed current (estimated at the end of the 250-msec voltage step) with an IC_{50} of 1.71 ± 0.27 μM (n = 14). In the absence of any other pharmacological agent, 50 μM loperamide produced a 97 ± 2% (range, 87–100%; n = 7) reduction of the control I_{Ba}; in five of these cells, a ≥97% reduction was observed (Figs. 5 and 6).

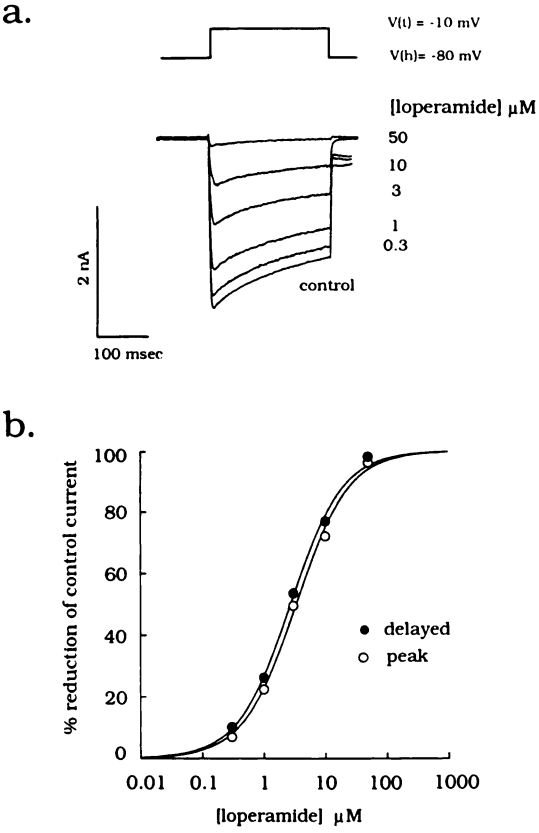

Fig. 5. Loperamide concentration-dependently blocks whole-cell I_{Ba} in cultured mouse hippocampal neurons. a, A 250-msec voltage step to −10 mV from a holding potential of −80 mV evoked a large inward I_{Ba}, which was slowly inactivating. Superfusion with loperamide, at the concentrations indicated to the *right of the traces*, reduced I_{Ba} in a concentration-dependent manner. Full block was achieved with 50 μM loperamide, with recovery to approximately 75% of control (data not shown). *Traces*, leak-subtracted currents. *Abscissa*, time (msec). *Ordinate*, current amplitude (nA). b, Dose-inhibition plot for loperamide as an antagonist of the I_{Ba} shown in a. Values indicate the percentage reduction of the control peak (○) and delayed (240 msec after peak) (●) I_{Ba} at each concentration of loperamide tested. In this neuron the IC_{50} values estimated (see Materials and Methods) were 3.4 and 2.7 μM, respectively, for peak and delayed currents; Hill coefficients were not significantly different from 1. *Abscissa*, loperamide concentration. *Ordinate*, percentage reduction of the control I_{Ba}.

In hippocampal pyramidal neurons, the whole-cell I_{Ba} evoked by voltage steps from a holding potential of −80 mV to a test potential of −10 mV has been shown to be composed of nifedipine-sensitive and nifedipine-resistant, ω-CgTx-sensitive components, with a variably sized residual component resistant to both, possibly reflecting P-type channel activity (15, 16, 29–31). In the present experiments, 10 μM nifedipine attenuated the whole-cell I_{Ba} by 23 ± 3% (range, 16–34%; n = 5), which on coapplication of 10 μM ω-CgTx increased to 80 ± 4% inhibition (range, 64–92%; n = 5) (Fig. 6b). In the same neurons 50 μM loperamide produced 85 ± 5% (range, 68–100%) reduction of the control I_{Ba}, suggesting that loperamide blocks multiple types of HVA Ca^{2+} channels. An example of the absence of selectivity in the action of loperamide among types of HVA Ca^{2+} channel can be seen in Fig. 6. The nifedipine-resistant component of the delayed I_{Ba} was completely blocked by loperamide, with an IC_{50} of 2.48 ± 0.59 μM (n = 9), a value similar to that seen in the absence of nifedipine (see above). In confirmation, 1 μM loperamide had similar effects on both control and nifedipine-resistant components of I_{Ba}, producing 31 ± 4% and 35 ± 4% (n = 11) reduction of control responses in the absence and presence of 10 μM nifedipine, respectively (Fig. 6).

Effect of loperamide on Ca^{2+} channel kinetics. Loperamide did not alter the rate of onset of the whole-cell I_{Ba} in any of the neurons tested, nor did it produce a significant shift in the current-voltage relationship for the I_{Ba} (n = 10; data not shown). In contrast to dihydropyridine Ca^{2+} channel antagonists, which are known to accelerate the inactivation of L-type Ca^{2+} channels (24, 35), there was no indication that loperamide affected the inactivation kinetics of the whole-cell I_{Ba}, having similar effects on both the peak and delayed components of the current (see Figs. 5–7). More extensive studies on the kinetics of the loperamide block of I_{Ba} were not attempted due to the difficulty of maintaining an adequate clamp of the membrane potential in a preparation of cultured neurons with extensive processes. Also in contrast to dihydropyridines, the action of loperamide was not voltage dependent. In 15 neurons tested, application of 3 μM loperamide produced 60 ± 3% reduction of the delayed I_{Ba} evoked from a holding potential of −80 mV to a test pulse of +10 mV, whereas when the membrane potential was set at a holding potential of −40 mV there was a 68 ± 4% reduction of the control I_{Ba} response by the same concentration of loperamide. An example of the lack of appreciable voltage dependence for the action of loperamide can be seen in Fig. 7.

The block of I_{Ba} by loperamide occurred rapidly, although not completely, during the first evoked current. Although apparently dependent on the opening of Ca^{2+} channels (see Fig. 7), the loperamide block of I_{Ba} continued to increase further even in the absence of channel activation. Furthermore, when currents were evoked at the higher frequency of 0.2 Hz rather than 0.05 Hz, there was only a small increase in the percentage inhibition produced by 3 μM loperamide (69 ± 6% and 66 ± 6% reductions at 0.2 and 0.05 Hz, respectively; n = 6), which may, in part, be attributed to the observed increase in current run-down at the higher frequency. An example of the rate of onset of block of I_{Ba} by 3 μM loperamide can be seen in Fig. 7.

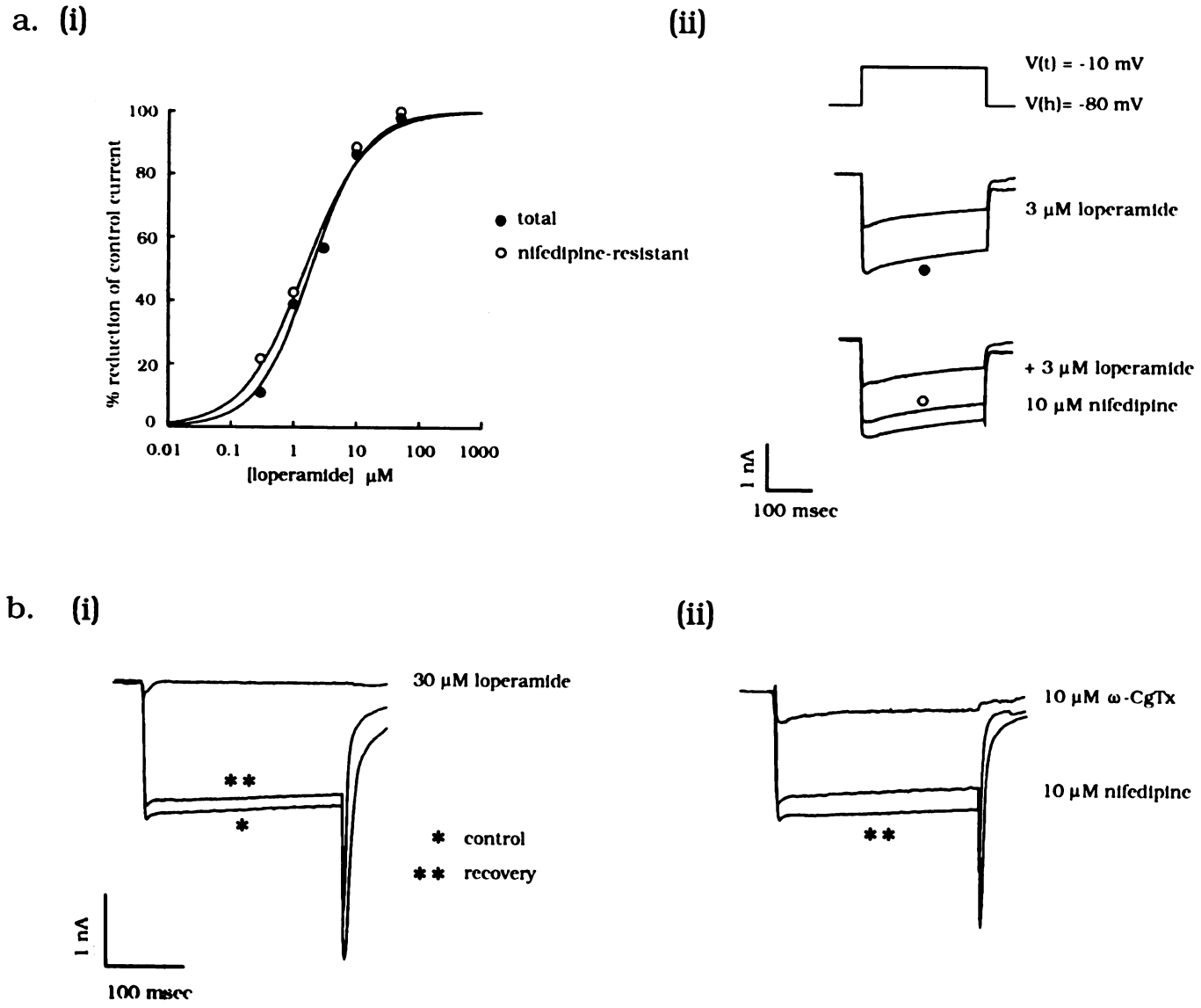

Fig. 6. Loperamide nonselectively blocks multiple subtypes of HVA Ca^{2+} currents. a, i, Dose-inhibition plots for loperamide as an antagonist of the whole-cell I_{Ba} (●) and of the current remaining after superfusion with 10 μM nifedipine (○). In the neuron tested here the IC_{50} values for loperamide were 1.9 and 1.5 μM in the absence and presence of nifedipine, respectively. *Abscissa*, concentration of loperamide. *Ordinate*, percentage reduction of the control I_{Ba}. a, ii, Representative leak-subtracted whole-cell I_{Ba}, evoked by the voltage step indicated *above the traces*, from the same neuron as in a, i. *Upper trace*, superfusion with 3 μM loperamide produced ~60% reduction of control I_{Ba} (●), with recovery represented as the new control in the lower trace. *Lower trace*, application of 10 μM nifedipine (○) produced ~25% reduction of I_{Ba}, which was reduced further by ~60% by coapplication of 3 μM loperamide. *Abscissa*, time (msec). *Ordinate*, amplitude of I_{Ba} (nA). *V(t)*, test potential; *V(h)*, holding potential. b, i, In a second neuron control I_{Ba} (*), evoked by the same voltage step as in a, ii, was eliminated on superfusion with 30 μM loperamide and almost full recovery was achieved with wash in control solution (**). b, ii, Subsequent superfusion with 10 μM nifedipine produced a 16% block of the whole-cell I_{Ba}, which was further increased to an 85% block by coapplication of 10 μM ω-CgTx. Some recovery was observed with washing (data not shown). *Abscissa*, time (msec). *Ordinate*, amplitude of I_{Ba} (nA).

Evidence that loperamide acts externally to produce the block of I_{Ba}. The voltage independence of the loperamide-induced block of I_{Ba} may indicate an intracellular target and, because loperamide is known to possess calmodulin antagonist properties (4), it was pertinent to test whether loperamide could block I_{Ba} when applied internally. Because I_{Ba} persisted when loperamide (10 μM) was included in the intracellular solution, it is unlikely that its I_{Ba}-blocking effects are mediated through interaction with calmodulin (see Ref. 36).

Loperamide blockade of NMDA-evoked currents. The action of loperamide on amino acid-evoked responses was investigated in 27 neurons. At a holding potential of −60 mV superfusion with loperamide (10–300 μM) produced a rapid block of steady state NMDA-evoked responses, with an IC_{50} of 73 ± 7 μM (n = 7) (Fig. 8). In the presence of 100 μM loperamide there was a 66 ± 6% (n = 6) reduction of control responses to 60 μM NMDA, with a smaller effect on control responses to 60 μM kainate and 10 μM AMPA (27 ± 3%, n = 9, and 14 ± 5%, n = 6, reduction of control, respectively). It is therefore likely that, at the concentrations used in subsequent experiments (50 or 60 μM), the action of loperamide was predominantly selective for the NMDA subtype of glutamate receptor. An example of the potency and selectivity of the action of loperamide can be seen in Fig. 8. The action of loperamide was rapid in onset and readily reversible upon wash with control solution, except at

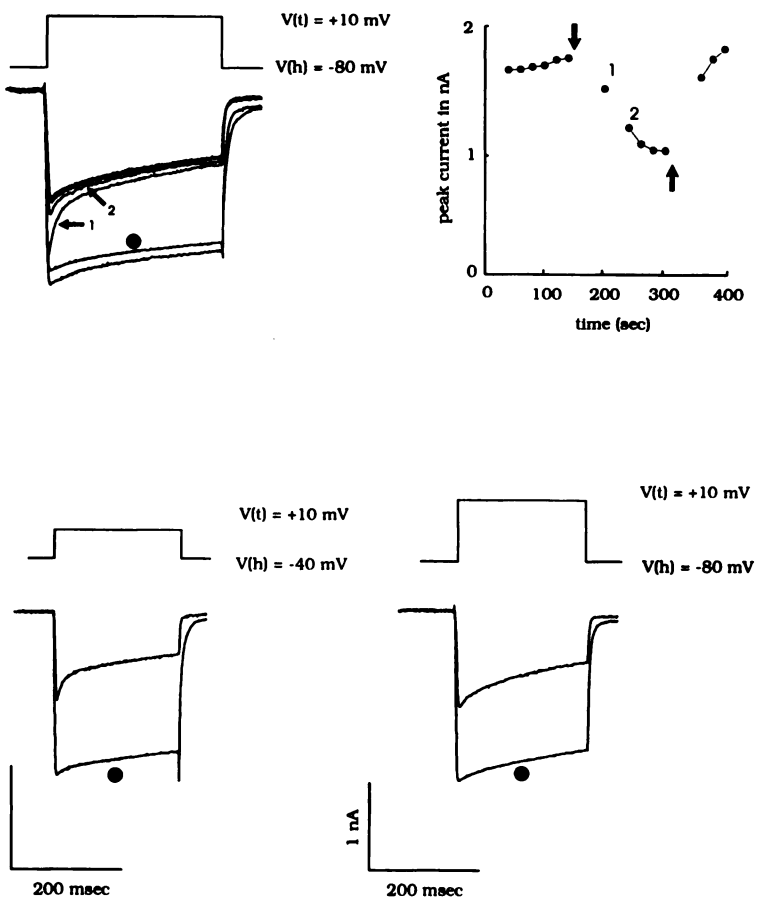

Fig. 7. Loperamide block of I_{Ba} is rapid and not dependent on membrane holding potential. a, *Left*, *traces*, rate of block of I_{Ba} by 3 μM loperamide. Control current (●) was rapidly blocked during the first current (*trace 1*) evoked 60 sec after the onset of superfusion with loperamide. Further block developed in the absence of current activation (*trace 2*, evoked 40 sec after *trace 1*). Steady state block was achieved after three subsequent activations of I_{Ba} at 20-sec intervals. Full recovery on wash was subsequently obtained (*lowest trace*). *Right*, graphic representation of the progression of block of the peak component of I_{Ba} by loperamide. *Arrows*, onset and offset of loperamide application; *numbers* correspond to the traces labeled on the *left*. *Abscissa*, time. *Ordinate*, amplitude of peak I_{Ba}. b, *Traces*, effect of 3 μM loperamide on I_{Ba} evoked using voltage steps from −40 mV (*left*) and −80 mV (*right*) to a test potential of +10 mV, in the same neuron as in a. In this neuron there was ~50% reduction of the peak control I_{Ba} (●) at either holding potential, whereas the delayed components were reduced by 67% and 62%, respectively. *Abscissa*, time (msec). *Ordinate*, current amplitude (nA).

concentrations of >100 μM, when recovery was often slow and incomplete.

The block of NMDA-evoked responses by loperamide was not voltage dependent, with the percentage reduction of the steady state current being unaltered over a range of holding potential from −80 mV to +60 mV ($n = 6$) (Fig. 9). Superfusion with 50 μM loperamide shifted the dose-response plot for NMDA to the right, without a change in the EC_{50} for NMDA but with a 64 ± 6% ($n = 6$) reduction of the maximum NMDA-evoked response (Fig. 9). Because the percentage reduction of NMDA-evoked responses was not dependent on the concentration of agonist applied (1–300 μM), loperamide is unlikely to interact competitively with the agonist binding site of the receptor.

Haloperidol has recently been shown to act at the glycine binding site associated with the NMDA receptor-channel complex (37). Given the structural commonalties between haloperidol and loperamide, we investigated the effect of elevating the extracellular glycine concentration on the loperamide block of NMDA-evoked currents. The percentage reduction of the steady state NMDA response upon application of 60 μM loperamide was not altered when the glycine concentration was elevated from 1 to 10 μM, with the respective values being 45 ± 5% and 50 ± 5% of controls ($n = 10$; not significantly different, Student's paired t test, $p < 0.05$). Furthermore, loperamide, in contrast to haloperidol (37), did not show differential potency on the peak and steady state components of the NMDA-evoked response (IC_{50} for peak current = 80 ± 5 μM, $n = 5$). The percentage reduction of NMDA-evoked currents by 60 μM loperamide was also unaffected by the addition of 100 μM spermine, with the respective inhibitions being 53 ± 6% and 52 ± 4% in the absence and presence of spermine ($n = 7$; not significantly different, Student's paired t-test, $p < 0.05$).

Discussion

Loperamide at low micromolar concentrations blocks high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ and Ca^{2+} channel currents in cultured hippocampal pyramidal neurons. In the microspectrofluorimetric studies using fura-2, pharmacological agents dissected the high-$[K^+]_o$-evoked rise in $[Ca^{2+}]_i$ into a large nifedipine-sensitive component and two smaller components sensitive to either ω-CgTx or funnel web spider venom. The fact that loperamide at concentrations of >20 μM was able to abolish high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ suggests that it acts as a broad-spectrum blocker of HVA Ca^{2+} channels. This possibility was confirmed in the electrophysiological studies, where loperamide blocked both nifedipine-sensitive and nifedipine-resistant, ω-CgTx-sensitive Ca^{2+} channel currents, with IC_{50} values similar to those found in the fura-2 studies. The effect of loperamide was rapid in onset, was reversible, and was neither naloxone-sensitive nor mimicked by morphine, indicating that it was not mediated by opioid receptor-dependent mechanisms. In addition, the observed effects of loperamide are unlikely to reflect nonspecific effects on membrane excitability, because

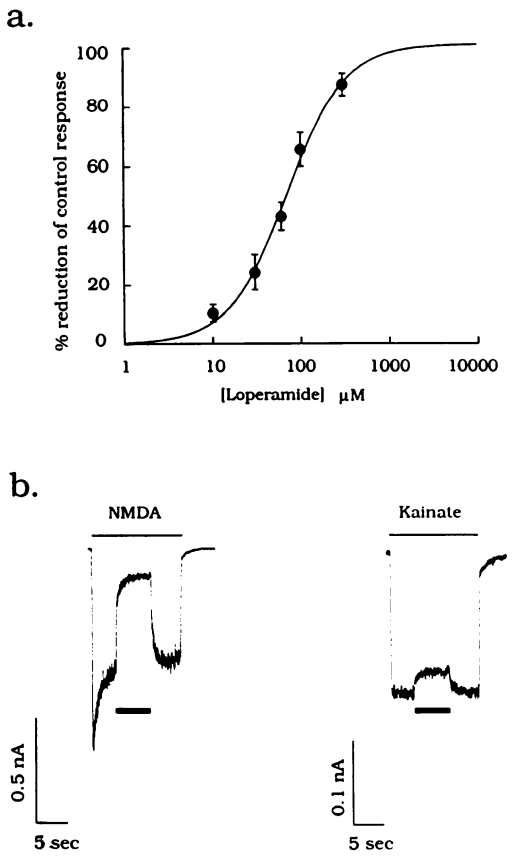

Fig. 8. Loperamide block of amino acid-evoked currents. a, Loperamide concentration-dependently attenuated responses evoked by 5-sec applications of 60 μM NMDA (see sample trace in b). Data points indicate mean ± standard error for seven neurons tested. Points were fit to a logistic equation with a computed IC_{50} value of 73 ± 7 μM and a Hill coefficient of 1.6 ± 0.2. *Abscissa*, concentration of loperamide. *Ordinate*, percentage reduction of control NMDA-evoked response. b, Sample traces of responses evoked by 60 μM NMDA (*left*) and 100 μM kainate (*right*) applied for the periods indicated by the *thin bars above the traces*. Loperamide (100 μM) (*thick bar below the traces*) attenuated the control NMDA-evoked response by 78% and the kainate-evoked response by 19%. *Abscissa*, time (sec). *Ordinate*, current amplitude (nA).

up to a concentration of 97.3 μM loperamide did not display local anesthetic activity with the partially desheathed frog sciatic nerve preparation (5). At concentrations approximately 30–40 times greater than those required to block HVA Ca^{2+} channels, loperamide attenuated NMDA-evoked currents. The block was not competitive in nature and was not due to interactions at either the strychnine-insensitive glycine binding site or the polyamine modulatory site on the NMDA receptor-channel complex. In addition, given that loperamide is charged at physiological pH, the voltage-independent nature of the noncompetitive NMDA antagonist action of loperamide may rule out a channel-blocking mechanism, although further studies are required to substantiate this possibility.

The mechanism whereby loperamide blocks HVA Ca^{2+} channels is unclear, but two possibilities, both of which have been implicated in its antidiarrheal actions (see Ref. 5), warrant discussion. These are the inhibition of calmodulin and a direct blockade of Ca^{2+} channels. Calmodulin antagonists, including chlorpromazine, trifluoperazine, and calmidazolium, have been shown to reduce Ca^{2+} currents in voltage-clamped snail neurons when applied extracellularly at concentrations in the 10–100 μM range (38). In contrast, lower concentrations (0.1–1.0 μM) of the same drugs increased the Ca^{2+} current in snail neurons (38), and HVA Ca^{2+} channel currents in hippocampal pyramidal neurons showed only an increase after bath application of trifluoperazine at a concentration associated with calmodulin antagonist activity (39). In the present experiments, loperamide was never observed to increase I_{Ba} or high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$ at any concentration tested. In addition, loperamide displays only relatively weak calmodulin antagonist activity, with IC_{50} values ranging from 75 to 100 μM (compared with 0.2–0.9 μM for calmidazolium and 12–14 μM for trifluoperazine) (4). Thus, Ca^{2+} channel blockade by loperamide occurs at concentrations substantially less than those associated with calmodulin antagonist activity. Furthermore, internal application of loperamide had little if any effect on I_{Ba}, arguing against an intracellular action. Inhibition of calmodulin is unlikely to play a direct role in the Ca^{2+} channel-blocking actions of loperamide (see Ref. 3).

Alternatively, loperamide may act directly on Ca^{2+} channels to modulate their activity. Loperamide inhibits the binding of [^{3}H]nitrendipine to guinea pig brain membranes with an IC_{50} of 10 μM (3), a result in accordance with the block of nifedipine-sensitive responses seen in the present experiments. Nevertheless, differences are discernible between the effects of dihydropyridines and loperamide. Thus, in contrast to loperamide, nifedipine failed to block all components of I_{Ba} and high-$[K^+]_o$-evoked rises in $[Ca^{2+}]_i$. Furthermore, the Ca^{2+} channel-blocking action of nifedipine is both voltage and frequency dependent and nifedipine enhances the inactivation of L-type Ca^{2+} channels (i.e., hastens the decay of the Ca^{2+} current) (24). In contrast, loperamide suppresses I_{Ba} without affecting its voltage dependence or inactivation time course. In addition, the effect of loperamide was not appreciably dependent on stimulation frequency and could continue to develop in the absence of channel activation. The observed onset of loperamide block during the first evoked current suggests, however, that channel opening may be required but that the kinetics of block may be more rapid than our present experimental protocol can resolve. An alternative possibility is that loperamide may act to inhibit Ca^{2+} channel activity in a manner reminiscent of that of antagonists of the phenylalkylamine class (e.g., verapamil) (20, 40). Thus, loperamide is able to reverse tiapamil-elicited lowering of [^{3}H]nitrendipine binding in guinea pig brain membranes (IC_{50} = 0.4 μM) (3) and verapamil can inhibit neuronal responses mediated by Ca^{2+} flux through dihydropyridine-insensitive Ca^{2+} channels (e.g., see Refs. 27 and 41). Interestingly, DXM, like verapamil, can also modulate Ca^{2+} channel activity by acting at a site that is allosterically coupled to the dihydropyridine binding site (Ref. 8; see also Ref. 42). Furthermore, a variety of dihydropyridine-resistant responses have been reported to be sensitive not only to toxins isolated from funnel web spider venom (e.g., see Refs. 31 and 43) but also to DXM, which inhibits K^+-evoked synaptosomal $^{45}Ca^{2+}$ influx (7), reduces both high-$[K^+]_o$-evoked Ca^{2+} influx into cultured neocortical neurons and excitatory amino acid release from hippocampal slices (41), and blocks both L- and N-type HVA Ca^{2+} channels in cultured cortical neurons in a voltage-independent manner, with IC_{50} values of 50–80 μM (10). In light of the similarities of action between DXM and loperamide in the periphery (see the introduction), together with the ability of loperamide to block not only nifedipine-sensitive but also

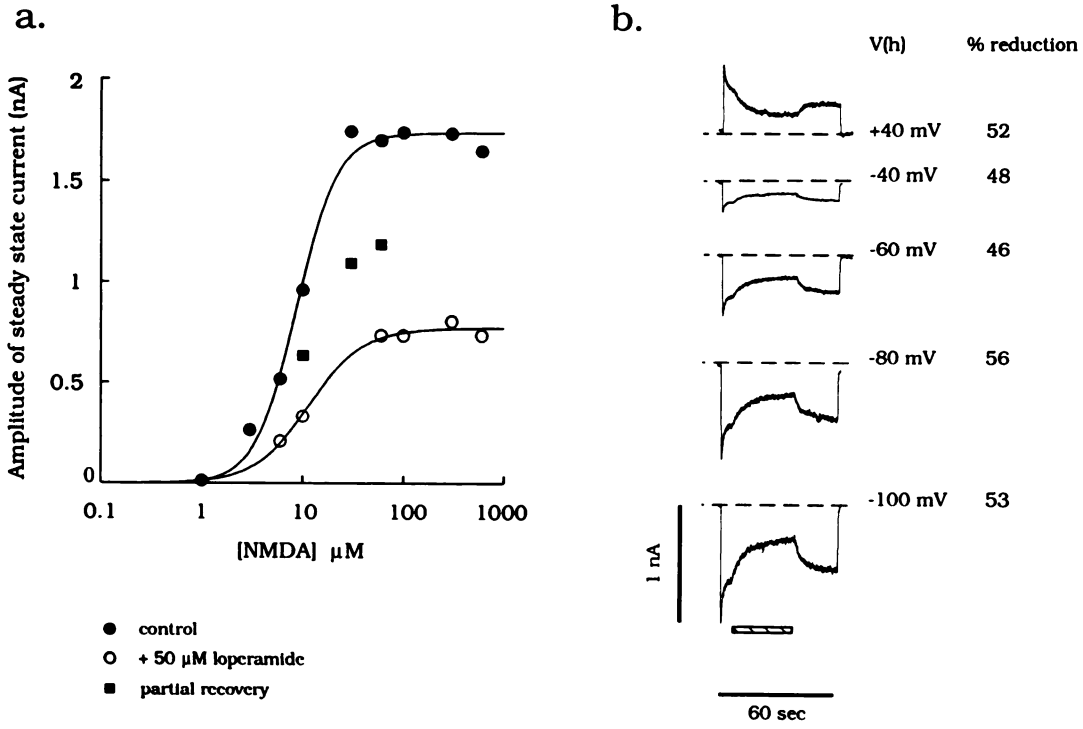

Fig. 9. Loperamide blocks NMDA-evoked responses in a noncompetitive and voltage-independent manner. a, A concentration-response plot for NMDA (●) was shifted to the right in the presence of 50 μM loperamide (○), with no change in the EC_{50} value for NMDA (6.5 μM and 7.5 μM in the absence and presence of loperamide, respectively) but with a 50% reduction of the maximum response amplitude. The effect of loperamide was partially reversed by washing in control solution (■). *Abscissa*, concentration of NMDA. *Ordinate*, amplitude of steady state NMDA current. b, Responses to 60-sec applications of 60 μM NMDA (*lower thin bar*) were evoked over a range of membrane holding potentials [*V(h)*]. The block of NMDA-evoked currents by 50 μM loperamide (applied for the period indicated by the *hatched bar*) was unaltered by changes in membrane potential, as indicated by values of percentage reduction to the *right of the traces*. *Time bar*, 60 sec. *Ordinate*, current amplitude (nA).

nifedipine-resistant responses, it would be of interest to examine the ability of loperamide to block Ca^{2+} channel-dependent neuronal responses that are insensitive to dihydropyridines and/or ω-CgTx. In addition, given recent reports that selective blockers of both L- and N-type Ca^{2+} channels possess neuroprotective properties (e.g., Refs. 44 and 45), an examination of the neuroprotective efficacy of a broad-spectrum blocker of HVA Ca^{2+} channels such as loperamide (or blood/brain barrier-permeant analogs) (1) may prove worthwhile.

There are experimental circumstances in which a complete and readily reversible blockade of neuronal HVA Ca^{2+} channels might be advantageous. In this regard, the use of combinations of selective organic blockers poses practical difficulties including, notably, light sensitivity and marked voltage dependence (dihydropyridines) (24, 28, 35) and expense and relative lack of reversibility (spider and marine snail toxins). Furthermore, whereas polyvalent cations such cadmium produce a relatively nonselective block of voltage-activated Ca^{2+} channels (16, 25, 28), their effects are often not easily reversed and their use is precluded in experiments employing, for example, high pH media (due to the formation of insoluble salts) or the Ca^{2+}-sensitive dye fura-2 (which possesses high affinity for polyvalent cations other than Ca^{2+}) (12, 32, 46). Loperamide appears to be devoid of these practical difficulties and may prove to be a useful tool for experiments in which a general suppression of multicomponent HVA Ca^{2+} channel currents is required.

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