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Maitotoxin-Elevated Cytosolic Free Calcium in GH_4C_1 Rat Pituitary Cells Nimodipine-Sensitive and -Insensitive Mechanisms

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ABSTRACT. Maitotoxin induces an extracellular Ca^{2+}-dependent membrane depolarization predominantly via activation of L-type voltage-dependent Ca^{2+} channels (L-VDCC) in GH_4C_1 rat pituitary cells. In contrast to studies employing intracellular dyes, electrophysiological studies have indicated that maitotoxin activates voltage-independent conductances. In the present study, we used fura-2 calcium digital analysis to investigate the actions of very low concentrations of maitotoxin on cytosolic free calcium ($[Ca^{2+}]_i$) in GH_4C_1 cells in an effort to distinguish different calcium entry mechanisms. Maitotoxin at concentrations as low as 0.01 ng/mL elevated $[Ca^{2+}]_i$ 35 ± 3% and induced membrane depolarization. The concentration dependency for maitotoxin-elevated $[Ca^{2+}]_i$ was biphasic with the first phase maximal at 0.05 to 0.5 ng/mL and the minimum EC_{50} of the second phase about 2.0 ng/mL. Nimodipine (100 nM), a dihydropyridine antagonist of L-VDCC, prevented the $[Ca^{2+}]_i$ increase and depolarization induced by up to 0.1 ng/mL maitotoxin, but not at higher concentrations (0.5 ng/mL) of maitotoxin. This indicates that lower concentrations (0.1 ng/mL) of maitotoxin require L-VDCC, whereas higher concentrations (≥ 0.5 ng/mL) of maitotoxin may require additional ionic mechanisms. Maitotoxin (0.5 ng/mL) induced $^{45}Ca^{2+}$ uptake and depolarization in L^{tk-} cells which lack VDCC. Reducing extracellular Cl^- from 123 to 5.8 mM increased the magnitude of membrane depolarization by maitotoxin (0.5 ng/mL), which suggests that a Cl^- conductance participates in depolarization induced by higher maitotoxin concentrations. Taken together, our results indicate that maitotoxin activates at least two ionic mechanisms. At lower concentrations of maitotoxin, the primary ionic mechanism requires the activation of L-VDCC; however, at higher maitotoxin concentrations, additional ionic mechanisms are involved in the entry of extracellular Ca^{2+}. This latter mechanism may represent the voltage-independent pathway evident under voltage clamp conditions. BIOCHEM PHARMACOL 51;6:759–769, 1996.

KEY WORDS. maitotoxin; L-VDCC; intracellular Ca^{2+}; membrane depolarization; ionic conductances

Maitotoxin, a water-soluble polyether, has long been recognized as a Ca^{2+} channel activator [1]. The pharmacologic activity of maitotoxin has been studied in numerous systems and is generally shown to activate two pathways in the cell: calcium influx and phosphatidylinositol hydrolysis [2, 3]. However, the precise mechanisms involved in maitotoxin elevation of $[Ca^{2+}]_i$ remain unknown. Maitotoxin stimulates Ca^{2+} influx in many, but not all cell types [4]. Several hypotheses exist regarding the mechanism of Ca^{2+} influx induced by maitotoxin. First, maitotoxin exerts its action primarily through VDCC†. This has been suggested since organic antagonists of L-type and N-type calcium channels can inhibit Ca^{2+} influx induced by maitotoxin in neuroblastoma and PC12 cells [4, 5]. Second, VDCC are not directly activated by maitotoxin; rather, they are activated as the result of depolarization induced by the entry of Ca^{2+} or other mono- or divalent cations through maitotoxin-sensitive, nonselective cation channels. This has been suggested since dihydropyridines, antagonists of type L-VDCC, have little effect on maitotoxin-induced Ca^{2+} influx in smooth muscle BC3H1 cells and synaptosomes [6, 7]. Finally, it has been suggested that the primary target of maitotoxin is a receptor-mediated calcium channel, because SK&F 96365, an inhibitor of receptor-mediated calcium entry, prevents Ca^{2+} influx induced by maitotoxin [8]. Maitotoxin appears not to be an ionophore since it does not cause Ca^{2+} influx in liposomes and mitochondria [9].

Maitotoxin induces membrane depolarization in many cell systems [5, 7, 10, 11]. This effect is dependent on extracellular Ca^{2+}. In PC12 cells, it has been shown that maitotoxin-induced depolarization is dependent on the influx of Ca^{2+} through VDCC, since this effect is inhibited by nicardipine and ω-CgTx, antagonists of VDCC [5]. In GH_4C_1 cells, we

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† *Abbreviations:* VDCC, voltage-dependent calcium channels; HBBS, HEPES-buffered balanced salt solution; and DMEM, Dulbecco's Modified Eagle's Medium.

have shown that the predominant effect of maitotoxin on depolarization is dependent upon activation of L-VDCC [10]. However, because nimodipine did not block maitotoxin-induced depolarization completely, we could not rule out entirely the possibility that the initial action of maitotoxin may involve other ion currents. By contrast, electrophysiological studies [12–17] have indicated that maitotoxin induces large inward currents that are voltage independent. Although the conducting ion(s) reported for these currents has differed between reports, the conductance appears not to be highly selective. The nonselective nature of maitotoxin-induced currents is supported by radioisotope uptake studies, indicating that maitotoxin increases the influx of many ions ($^{86}Rb^+$, $^{22}Na^+$, $^{54}Mn^+$ and $^{36}Cl^-$) in addition to Ca^{2+} [9].

In the present study, we used fura-2 calcium digital analysis to investigate the actions of low concentrations of maitotoxin on cytosolic free calcium ($[Ca^{2+}]_i$) in GH_4C_1 cells. Our results indicate that maitotoxin-elevated $[Ca^{2+}]_i$ is a biphasic event. At very low concentrations of maitotoxin, L-VDCC were required for both membrane depolarization and elevated $[Ca^{2+}]_i$. At higher concentrations ($\geq$ 0.5 ng/mL), maitotoxin activated additional ionic mechanisms that were insensitive to nimodipine.

MATERIALS AND METHODS

Materials

Maitotoxin was purchased from Wako Pure Chemicals Ltd. (Richmond, VA). Other reagents were purchased from the following sources: $^{45}CaCl_2$ was from New England Nuclear (Boston, MA). Scintiverse scintillation fluid was obtained from Fisher Scientific (Norcross, GA). Ham's F10 nutrient mixture and horse and fetal bovine serum were purchased from Grand Island Biological (Grand Island, NY); 96-well tissue culture microplates were from Costar (Cambridge, MA); bisoxonol and fura-2 were from Molecular Probes (Eugene, OR); sodium isethionate was obtained from the Eastman Kodak Co. (Rochester, NY). Nimodipine was provided by Dr. A. Scriabine of Miles Pharmaceuticals (West Haven, CT).

Cell Culture

Stock GH_4C_1 cultures were maintained in Ham's Nutrient Mixture supplemented with 15% horse and 2.5% fetal bovine serum (F10+) in the absence of antibiotics for a maximum of 10 passages [18]. Stock cultures were treated with 0.01% EDTA-phosphate-buffered saline to detach cells from the dishes.

Calcium Digital Imaging

The Ca^{2+}-sensitive indicator dye fura-2 was used to monitor changes in intracellular calcium in individual cells. Cultured cells were loaded with fura-2/AM (5 μM) for 30 min at 37°. Cells were placed into a chamber and visualized on a color monitor with an Axiovert 35 inverted research microscope interfaced with an Attofluor Ratio Vision digital fluorescence imaging system. The system uses an intensified CCD camera and a 486DX2 computer system to acquire images of the cells at 334 and 380 nm automatically. Ratios were taken for the data at each wavelength (334/380 nm) and quantified for designated sample areas within the cell. Background fluorescence was measured from a cell-free region of the coverslip. All experiments were done in Mg^{2+}-free HBBS (20 mM HEPES, pH 7.2, 10 mM glucose, 118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$) at room temperature. Calcium calibrations were performed using *in vitro* standards [19].

Measurement of Membrane Potential

Changes in the membrane potential of GH_4C_1 cells were monitored with the fluorescent dye bisoxonol. Bisoxonol is a permeant lipophilic anion whose distribution across the cell membrane is dependent upon the membrane potential. Fluorescence emission of this dye increases with membrane depolarization. The procedure is based on that described by Koch *et al.* [20]. Briefly, cells were allowed to equilibrate for 30 min at room temperature in HBBS, washed, and resuspended at a concentration of 2–4 × 10^6 cells/mL in HBBS without bovine albumin. Bisoxonol was added to cells at a final concentration of 20 nM, and the fluorescence (excitation wavelength = 540 nm, emission wavelength = 580 nm) was monitored in a well-stirred cuvette wtih an SLM 8000 spectrofluorometer (from SLM Amincojluc). The excitation and emission slits were 4 and 8 nm, respectively, and resulted in no significant signal from scattered light.

$^{45}Ca^{2+}$ Uptake Assay

Cells were plated in 0.1 mL F10+ at 0.3 × 10^6 cells/mL in 96-well tissue culture plates and allowed to attach overnight. F10+ medium was then removed and replaced with HBBS containing 5 μCi/mL $^{45}Ca^{2+}$ with or without treatments and incubated for 5 min [21]. The assay was terminated by removal of the $^{45}Ca^{2+}$-containing medium and rinsing with ice-cold HBBS, followed by addition of 0.1 mL scintillation fluid. The cells were lysed with scintillation fluid for 1 hr, and the plate was then counted directly using a microplate scintillation counter.

RESULTS

Concentration Dependency of Maitotoxin Elevation of $[Ca^{2+}]_i$

Maitotoxin has been reported to elevate $[Ca^{2+}]_i$ in many cell types although the initial mechanisms of maitotoxin action are still unclear. We previously investigated maitotoxin action to induce membrane depolarization in GH_4C_1 cells and determined that depolarization is a calcium-dependent process that is inhibited, in large part, by the L-VDCC antagonist nimodipine [10]. Here, we investigated the actions of maitotoxin to elevate $[Ca^{2+}]_i$ using fura-2 single cell calcium image analysis. Maitotoxin elevated $[Ca^{2+}]_i$ at concentrations substantially lower (0.01 ng/mL) than previously determined, and the con-

centration-response curve was biphasic (Fig. 1). The first phase was maximum at between 0.05 and 0.5 ng/mL; the second phase had an estimated minimum EC_{50} of 2.0 ng/mL. Maitotoxin was not tested at higher concentrations since 10 ng/mL decreased cell viability (unpublished data). The onset of maitotoxin-elevated $[Ca^{2+}]_i$ paralleled the onset of depolarization that we reported previously and was slow by comparison with that elevated in response to K^+-induced depolarization (Fig. 2). Maitotoxin-elevated $[Ca^{2+}]_i$ paralleled the onset of depolarization that we reported previously. We next examined the requirement for L-VDCC for both low and high concentrations of maitotoxin-elevated $[Ca^{2+}]_i$.

Involvement of L-VDCC in Maitotoxin-Induced $[Ca^{2+}]_i$ *Increase*

Previous work indicated that nimodipine largely, but not completely, inhibited maitotoxin-induced membrane depolarization in GH_4C_1 cells. By calcium single cell analysis, we determined that nimodipine (100 nM) pretreatment prevented elevated $[Ca^{2+}]_i$ by maitotoxin given at 0.1 ng/mL, but not at higher concentrations (0.5 or 5.0 ng/mL) (Fig. 3A). $[Ca^{2+}]_i$ elevated by 0.1 ng/mL maitotoxin was also reversed by posttreatment with nimodipine (Fig. 3B). These findings indicate that the $[Ca^{2+}]_i$ increase induced by 0.1 ng/mL maitotoxin is the result of Ca^{2+} entry through L-VDCC. However, at higher concentrations, maitotoxin-elevated $[Ca^{2+}]_i$ may involve other ionic mechanisms. To further test the possibility that maitotoxin at high concentrations elevates $[Ca^{2+}]_i$ through a nimodipine-insensitive mechanism(s), we next examined the effect of nimodipine on maitotoxin-induced Ca^{2+} uptake. Fig-

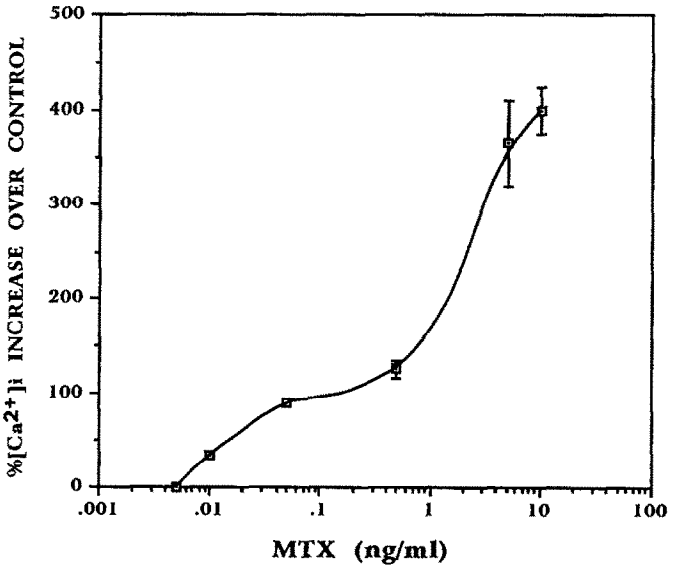


FIG. 1. Concentration dependency for maitotoxin-elevated cytosolic free calcium. Fura-2-loaded GH_4C_1 cells were treated with 0.005, 0.01, 0.05, 0.5, 5, or 10 ng/mL maitotoxin. The fluorescence from 30 cells was monitored simultaneously. Values are the means ± SEM of the percent increase above resting cytosolic free calcium (111.89 ± 9.6 nM) for five independent experiments.

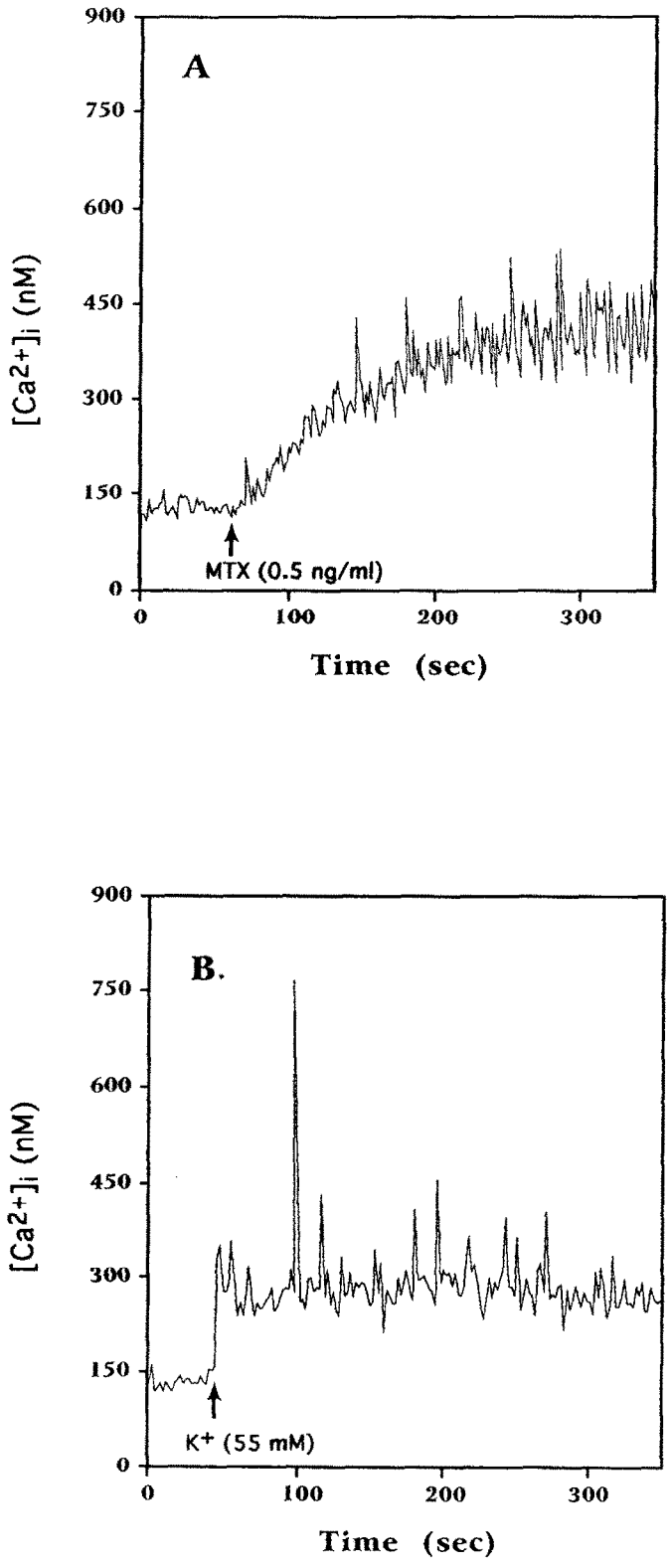


FIG. 2. Maitotoxin and high K^+-elevated cytosolic free calcium. Fura-2-loaded GH_4C_1 cells were treated with 0.5 ng/mL maitotoxin (panel A) or 55 mM K^+ (panel B). The fluorescence from 30 cells was monitored simultaneously. The fluorometric trace represents the average of 30 cells from a representative of three independent experiments.

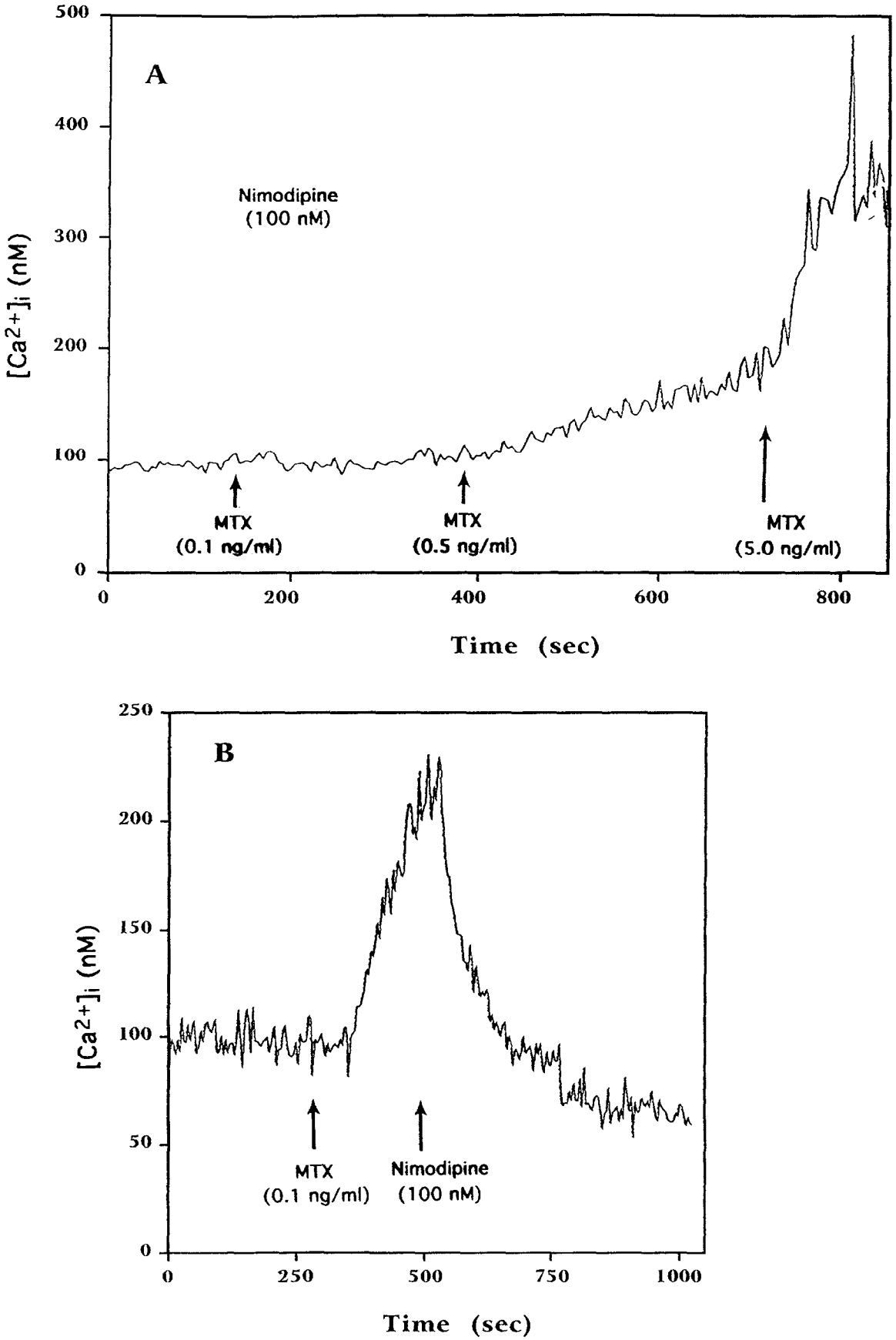


FIG. 3. Effect of nimodipine on maitotoxin-elevated cytosolic free calcium. Fura-2-loaded GH_4C_1 cells were treated with 100 nM nimodipine prior to (panel A) or after (panel B) maitotoxin addition. Addition of maitotoxin is marked with arrows. The fluorometric trace represents the average of 30 cells from a representative of three experiments.

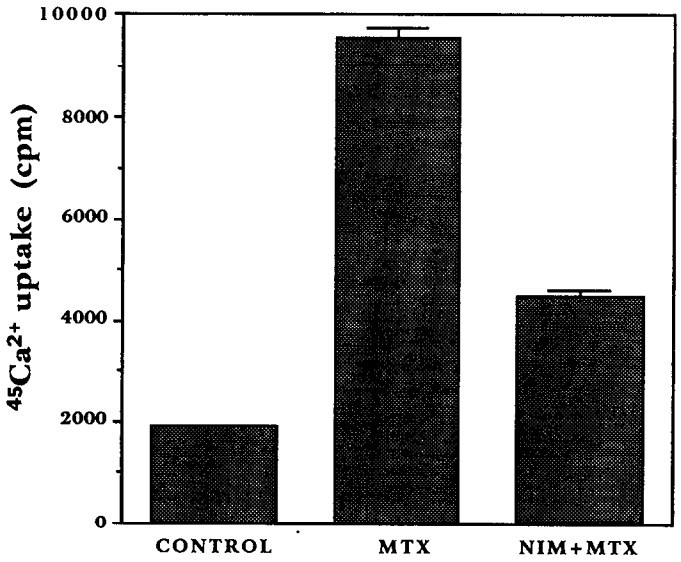


FIG. 4. Effect of maitotoxin on $^{45}Ca^{2+}$ uptake. Cells were plated in 0.1 mL F10+ at 0.3 × 10^6 cells/mL in 96-well tissue culture plates and allowed to attach overnight. Then F10+ medium was removed and replaced with HBBS containing 5 μCi/mL $^{45}Ca^{2+}$ with or without 5.0 ng/mL maitotoxin in the presence or absence of nimodipine (2 μM) for 5 min. Values are the means ± SEM for three independent experiments. Significance was determined by one-way analysis of variance ($P < 0.05$ for MTX vs control and NIM + MTX vs control).

ure 4 shows that maitotoxin at 5.0 ng/mL induced $^{45}Ca^{2+}$ uptake, and this effect was reduced only partially (57.4 ± 4.7%) with 2 μM nimodipine. We next examined the effects of low concentrations of maitotoxin on membrane depolarization.

Involvement of L-VDCC in Maitotoxin-Induced Depolarization

To further define the mechanisms of L-VDCC activation by maitotoxin, we investigated the action of nimodipine on maitotoxin-induced depolarization. Maitotoxin induced a depolarization at concentrations as low as 0.01 ng/mL (Fig. 5). Nimodipine at 100 nM prevented the depolarization induced by maitotoxin at 0.1 ng/mL, but not at 0.5 ng/mL or higher concentrations (Fig. 6A). Nimodipine given after maitotoxin-induced membrane depolarization slowly reduced the effects of maitotoxin (Fig. 6B). These results indicate that at lower concentrations (0.1 ng/mL) maitotoxin-induced depolarization was the result of L-VDCC activation, but at higher concentrations (0.5 ng/mL) other ion channels are probably involved. We next directed our investigation to the nimodipine-insensitive mechanism of maitotoxin-elevated $[Ca^{2+}]_i$.

Maitotoxin Effect on L^{tk-} Cells

To further test the hypothesis that maitotoxin can directly activate ion channels other than L-VDCC in GH_4C_1 cells, we examined the effect of maitotoxin on L^{tk-} cells which lack voltage-dependent calcium channels [22]. Maitotoxin at 0.5 ng/mL increased Ca^{2+} influx 34.2 ± 3.1% over the control, as measured by a $^{45}Ca^{2+}$ uptake assay (Fig. 7A). In addition, maitotoxin at 0.5 ng/mL induced membrane depolarization in these nonexcitable cells (Fig. 7B). The Ca^{2+} uptake and membrane depolarization could not be prevented by nimodipine. These results support the hypothesis that one of the targets for maitotoxin elevating $[Ca^{2+}]_i$ involves nimodipine-insensitive Ca^{2+} conductances.

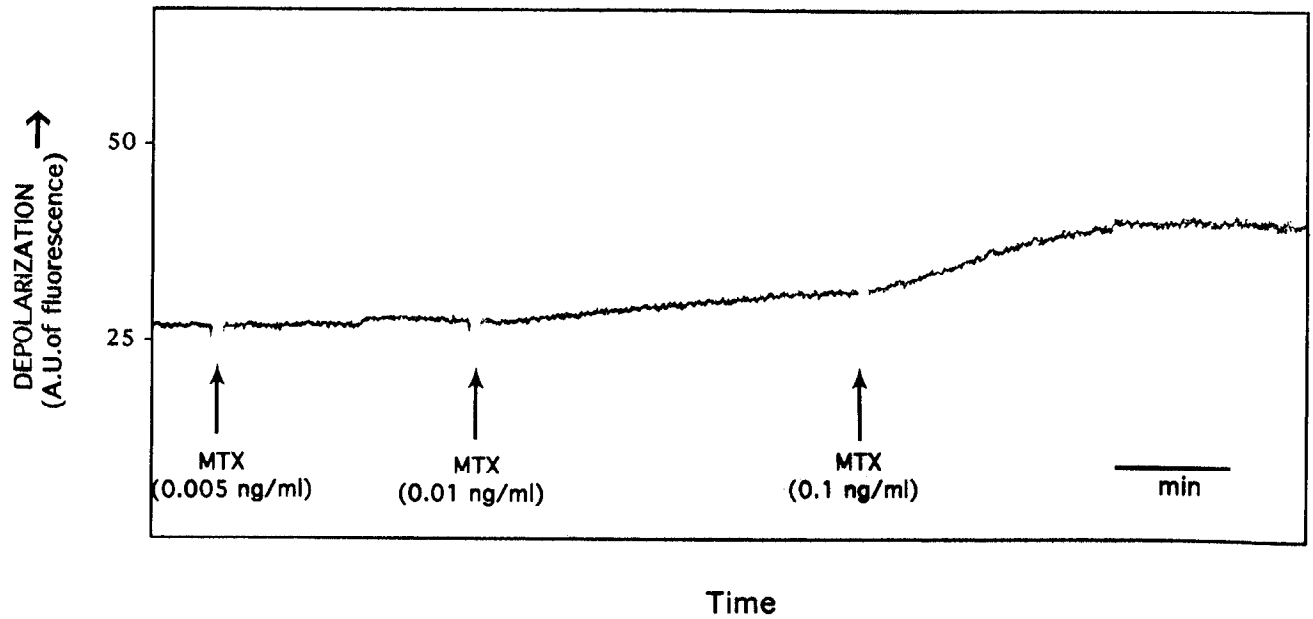
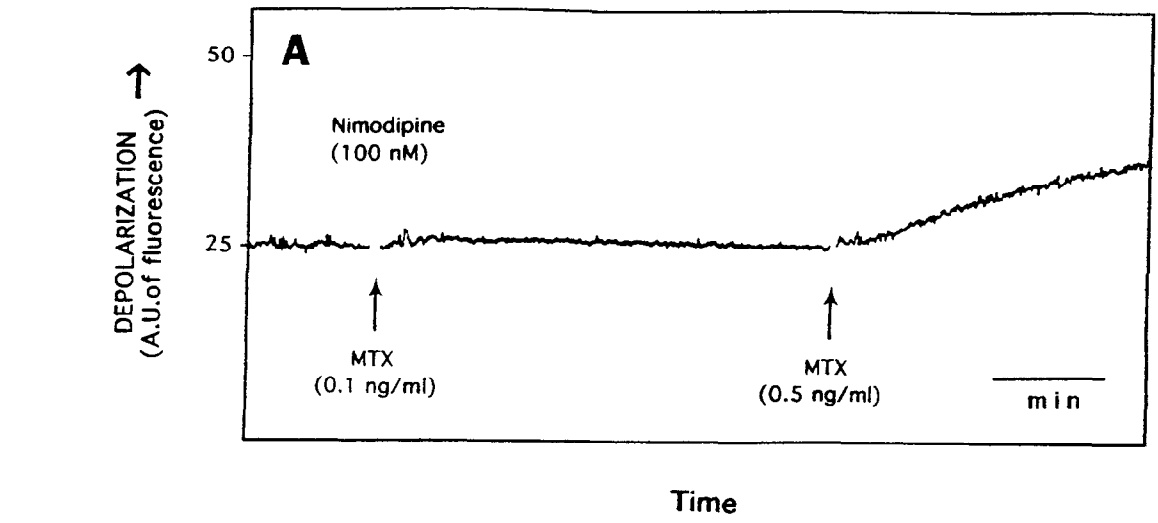


FIG. 5. Effect of maitotoxin on membrane depolarization. GH_4C_1 cells (6 × 10^6 cells/3 mL) were equilibrated with a final concentration of 20 nM bisoxonol in HBBS. Once a steady baseline value was obtained (approximate 2 min), cell suspensions were treated with 0.005, 0.01, and 0.1 ng/mL maitotoxin. The tracing is from a representative experiment.



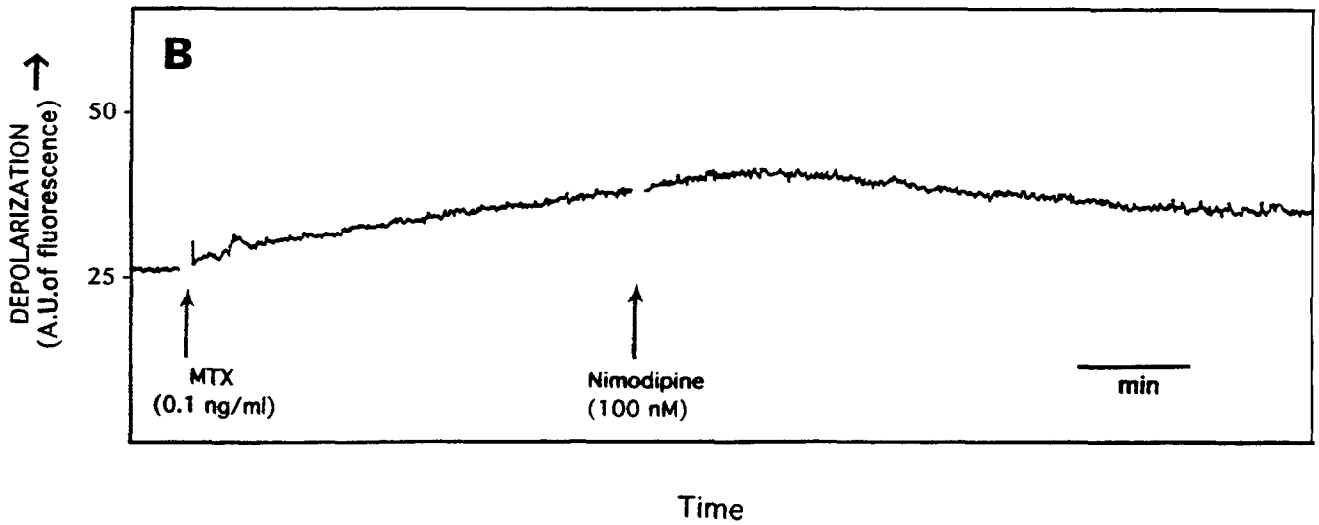


FIG. 6. Effect of nimodipine on maitotoxin-induced membrane depolarization. GH_4C_1 cells (6×10^6 cells/3 mL) were equilibrated with a final concentration of 20 nM bisoxonol in HBBS. Once a steady baseline value was obtained, cell suspensions were treated with 100 nM nimodipine prior to (panel A) or after (panel B) addition of maitotoxin. Addition of maitotoxin is shown with arrows. The tracing is from a representative of three experiments.

Effect of High Extracellular K^+ on Maitotoxin Action

We next investigated whether K^+-induced depolarization affects maitotoxin action on L-VDCC by examining the effect of high extracellular K^+ on maitotoxin action. Maitotoxin at 0.05 ng/mL did not further increase $[Ca^{2+}]_i$ in 55 mM K^+ medium (Fig. 8A); 0.5 ng/mL maitotoxin induced a small increase, whereas 5.0 ng/mL maitotoxin caused a large additional $[Ca^{2+}]_i$ increase in the presence of high K^+. Maitotoxin at 0.5 ng/mL also further depolarized cells in the presence of high K^+ (Fig. 8B). These results indicate that the effects of maitotoxin at 0.5 ng/mL and higher are largely independent of membrane potential or the activation state of L-VDCC.

Ionic Dependency of Maitotoxin-Induced Membrane Depolarization

Maitotoxin-induced depolarization required extracellular Ca^{2+} at all concentrations tested (data not shown). To examine

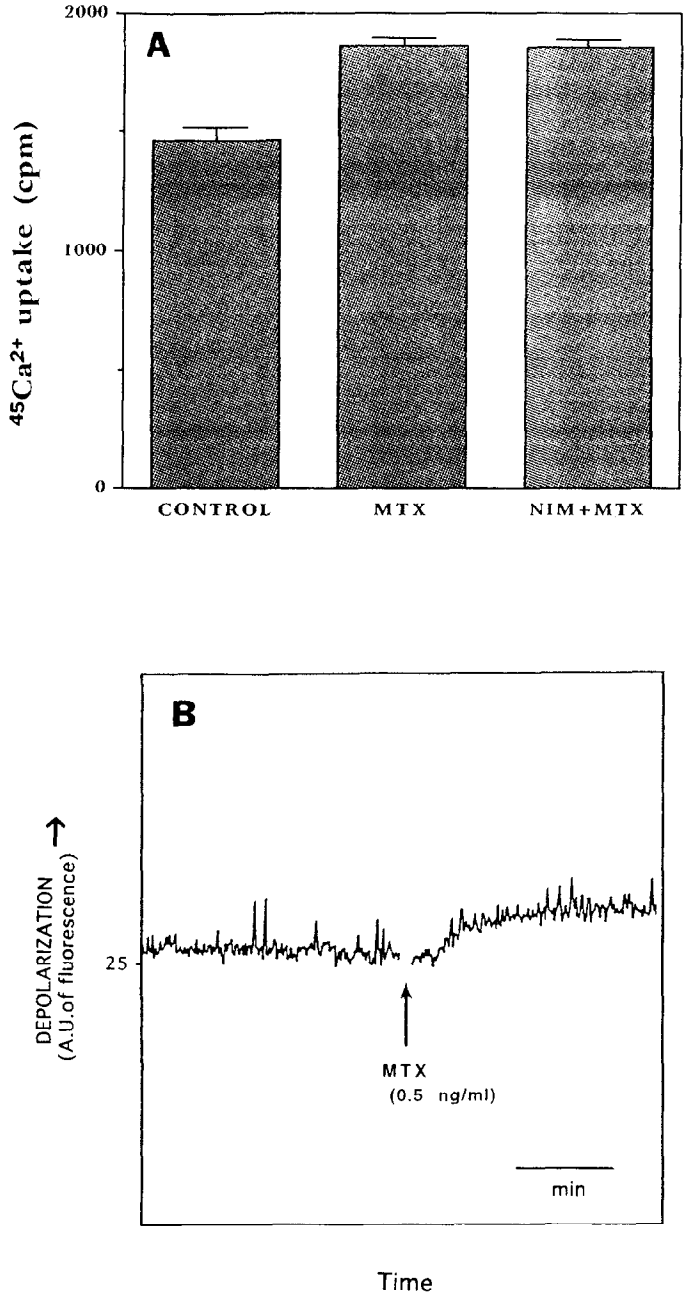


FIG. 7. Effect of maitotoxin on $^{45}Ca^{2+}$ uptake and membrane potential in L^{tk-} cells. In panel A, cells were plated in 0.1 mL DMEM at 0.3 × 10^6 cells/mL in 96-well tissue culture plates and allowed to attach overnight. DMEM was then removed and replaced with HBBS containing 5 μCi/mL $^{45}Ca^{2+}$ with or without 0.5 ng/mL maitotoxin in the presence or absence of nimodipine (2 μM) for 5 min. Values are the means ± SEM for four independent experiments. Significance was determined by one-way analysis of variance ($P < 0.01$ for MTX vs control and NIM + MTX vs control). In panel B, L^{tk-} cells (6 × 10^6 cells/3 mL) were equilibrated with a final concentration of 20 nM bisoxonol in HBBS. Once a steady baseline value was obtained, cell suspensions were treated with 0.5 ng/mL maitotoxin.

whether maitotoxin depolarized cells by increasing the Na^+ currents, we substituted choline chloride for sodium chloride. We found that this substitution did not affect depolarization induced by maitotoxin (data not shown). This indicates that Na^+ conductance is not the major ion involved in depolarization induced by maitotoxin. We next examined the Cl^- conductance involvement in maitotoxin-induced membrane depolarization. The intracellular chloride concentration is about 31 mM in GH_4C_1 cells and the Cl^- permeability is low [22]. The electric driving force for Cl^- is greater than its chemical driving force. Hence, if the Cl^- channel is activated, Cl^- efflux occurs and the membrane potential should be depolarized. To test the possibility of Cl^- conductance involvement in maitotoxin-induced depolarization, we decreased the $[Cl^-]_e$ from 123 to 5.6 mM by substituting sodium isethionate for sodium chloride. Isethionate is innocuous when used as a substitute anion for chloride [23]. The overall driving force for Cl^- in low $[Cl^-]_e$ medium is higher than for normal $[Cl^-]_e$ medium. The results in Fig. 9 show that low extracellular Cl^- did not affect membrane depolarization induced by lower concentrations of maitotoxin (0.05 ng/mL). However, at higher concentrations of maitotoxin (0.5 ng/mL), the magnitude of depolarization was increased with low $[Cl^-]_e$. This suggests that a Cl^- conductance is involved in membrane depolarization induced by 0.5 ng/mL maitotoxin.

DISCUSSION

This study investigated the ionic mechanisms of maitotoxin action to elevate $[Ca^{2+}]_i$. We found that maitotoxin induced a biphasic elevation of $[Ca^{2+}]_i$, which suggests the involvement of two mechanisms. Nimodipine, an antagonist of L-VDCC, prevented the $[Ca^{2+}]_i$ increase and membrane depolarization that was induced by lower concentrations ($\leq$ 0.1 ng/mL) of maitotoxin. This is consistent with an initial maitotoxin action that requires L-VDCC and that the depolarization is secondary to calcium entry. However, nimodipine did not prevent $[Ca^{2+}]_i$ increase and membrane depolarization induced by higher concentrations (> 0.5 ng/mL) of maitotoxin. These concentrations of maitotoxin also caused Ca^{2+} influx and membrane depolarization in cells (L^{tk-}) that lack L-VDCC. These results indicate that an ion conductance in addition to L-VDCC is involved in the action of higher concentrations of maitotoxin to elevate $[Ca^{2+}]_i$ and induce depolarization. The magnitude of depolarization induced by 0.5 ng/mL maitotoxin is increased with reduced extracellular Cl^-, suggesting that a Cl^- conductance may be involved in this nimodipine-insensitive mechanism.

Our previous results opened the possibility that the initial currents leading to maitotoxin-induced depolarization involve an ionic mechanism in addition to L-VDCC [10]. This is consistent with the reports of maitotoxin-induced voltage-independent ionic conductances in GH_4C_1 and other cell types [13–18]. In the present study, we found that maitotoxin at concentrations as low as 0.01 ng/mL elevated $[Ca^{2+}]_i$ in GH_4C_1 cells. This effect reached a plateau between 0.05 and 0.5 ng/mL. However, a second effect of maitotoxin to elevate

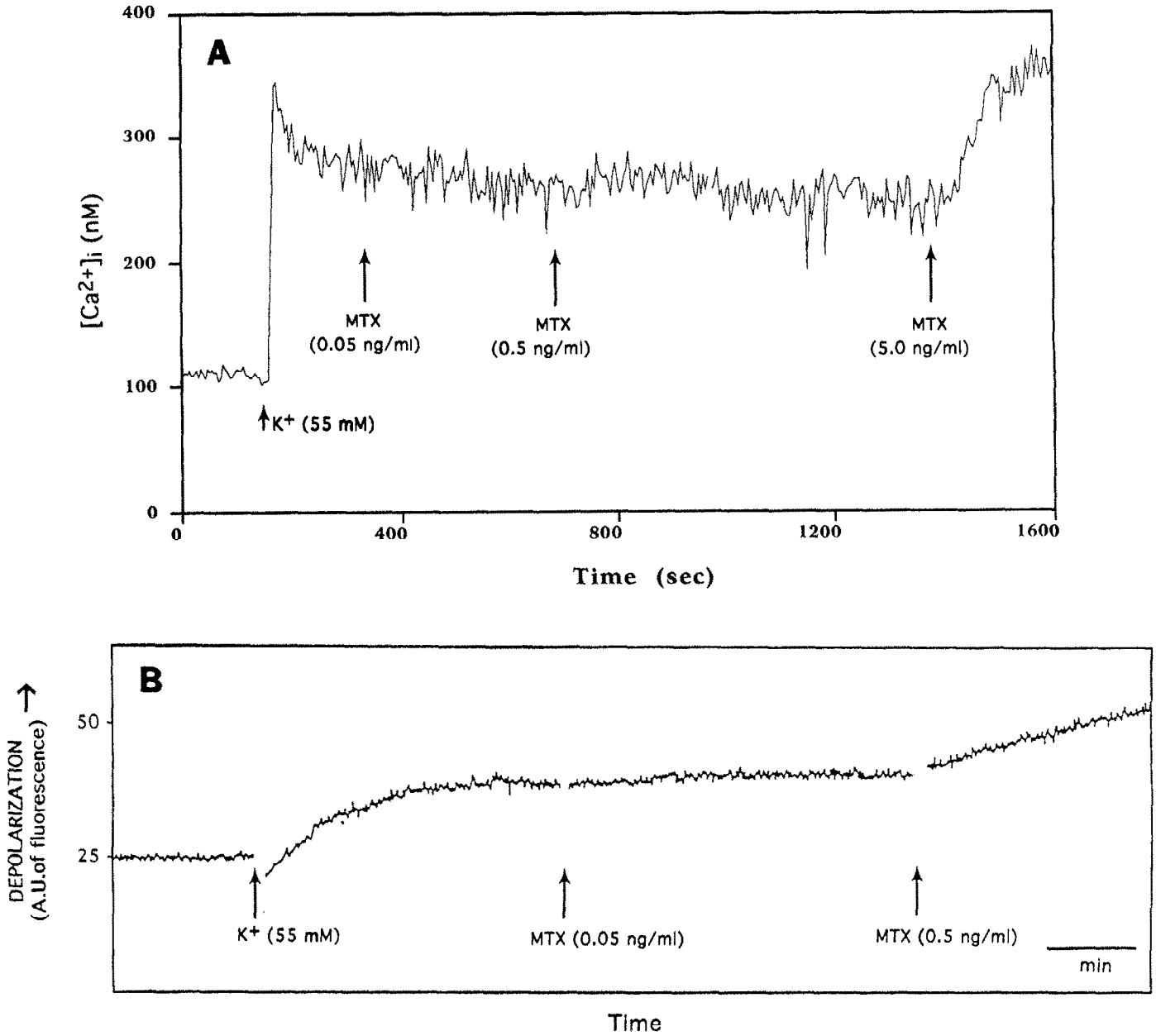


FIG. 8. High K^+ effect on maitotoxin-elevated cytosolic free calcium and induced depolarization. In panel A, fura-2-loaded GH_4C_1 cells were treated with 55 mM K^+. Once the K^+-elevated $[Ca^{2+}]_i$ reached a steady level, cells were treated with 0.05, 0.5, or 5.0 ng/mL maitotoxin. The fluorometric trace represents the average of 30 cells from a representative of three experiments. In panel B, GH_4C_1 cells (6×10^6 cells/3 mL) were equilibrated with a final concentration of 20 nM bisoxonol in HBBS. Once a steady baseline value was obtained, cells suspensions were treated with 55 mM KCl. Once the K^+-induced depolarization reached a steady level (about 3 min), cells were treated with 0.05 and 0.5 ng/mL maitotoxin.

$[Ca^{2+}]_i$ began between 0.1 and 0.5 ng/mL and increased up to 10 ng/mL. The biphasic increase of $[Ca^{2+}]_i$ suggests that two mechanisms are involved in maitotoxin-elevated $[Ca^{2+}]_i$. Since reduction of extracellular calcium fully prevents maitotoxin-elevated $[Ca^{2+}]_i$, it is likely that the initial action of maitotoxin to increase $[Ca^{2+}]_i$ results from entry of extracellular Ca^{2+}. However, we cannot discount that calcium may be necessary for an active conformation of maitotoxin or its binding to a target site.

Dihydropyridines have been reported to prevent maitotoxin-elevated $[Ca^{2+}]_i$ in some cell types, but only partially, or not at all, in other cell types [4–7]. We found that the inhibitory effect of nimodipine on maitotoxin-elevated $[Ca^{2+}]_i$ was dependent on the concentration of maitotoxin. Nimodipine prevented the action of lower concentrations of maitotoxin (< 0.5 ng/mL) to elevate $[Ca^{2+}]_i$, indicating Ca^{2+} entry through L-VDCC. Since membrane depolarization induced by 0.1 ng/mL maitotoxin was also abolished by nimodipine, this indicates that depolarization is secondary to activation of L-VDCC. This is consistent with the hypothesis that the primary target for maitotoxin elevation of $[Ca^{2+}]_i$ is the voltage-dependent Ca^{2+} channel. We previously reported that maito-

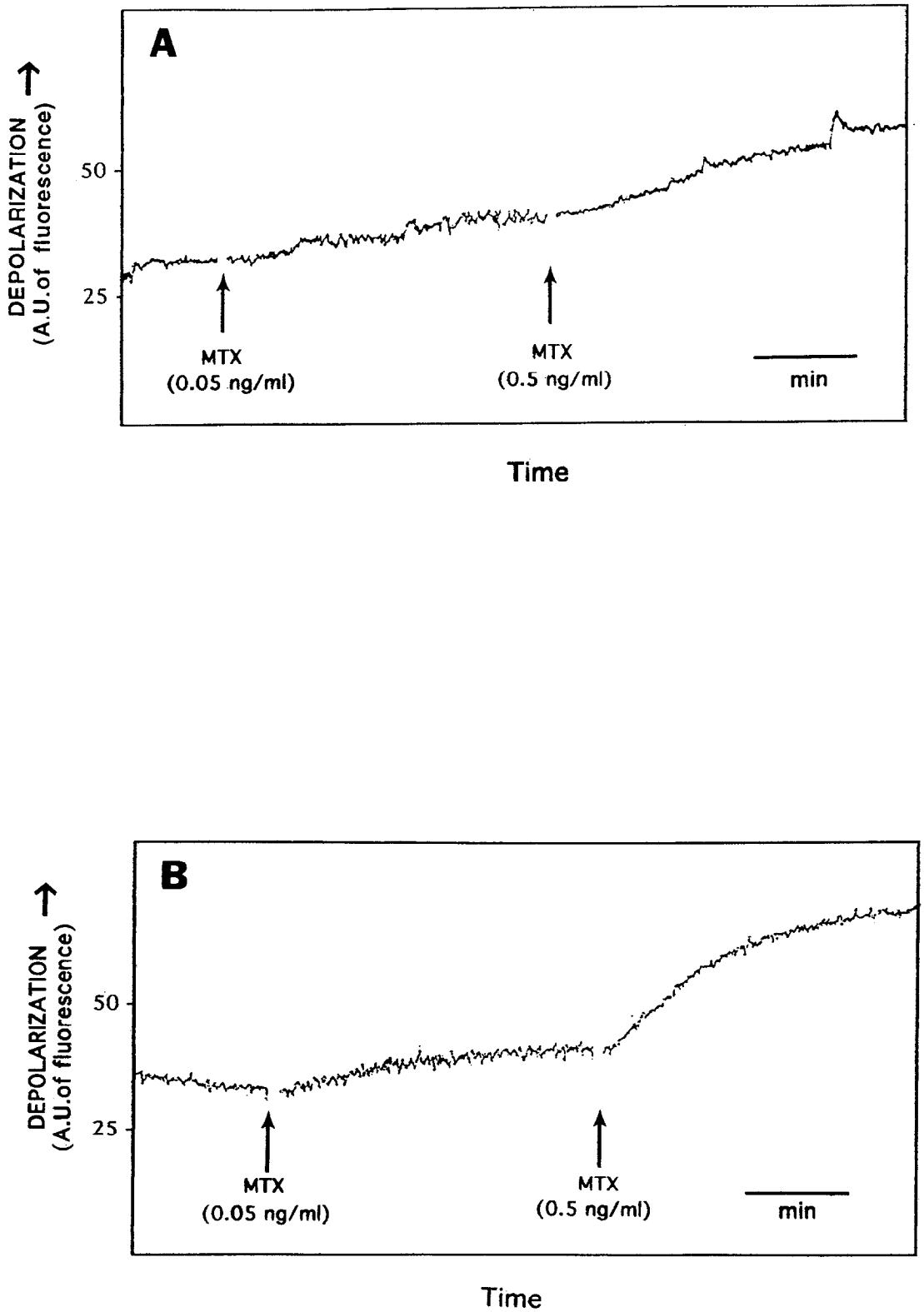


FIG. 9. Effect of extracellular Cl^- on maitotoxin-induced membrane depolarization. GH_4C_1 cells (6×10^6 cells/3 mL) were equilibrated with a final concentration of 20 nM bisoxonol in 123 mM (panel A) or 5.6 mM (panel B) Cl^- HBBS. Once a steady baseline value was obtained, cell suspensions were treated with 0.05 and 0.5 ng/mL maitotoxin.

toxin increases [^{3}H]PN 200-110 specific binding to intact GH_4C_1 cells without altering membrane potential, suggesting an interaction of maitotoxin at L-VDCC [10]. Voltage-dependent ionic channels are known to exist in three states: closed, open, and inactivated, each dependent on the membrane potential [23]. The action of maitotoxin at this lower concentration appears dependent upon membrane potential or the state of L-VDCC, since at 0.05 ng/mL maitotoxin did not induce an additional $[Ca^{2+}]_i$ increase or membrane depolarization in the presence of 55 mM K^+. Our data indicate that maitotoxin given at low concentrations leads to activation of L-VDCC to enhance calcium entry and induce membrane depolarization.

Our results show that higher concentrations (0.5 ng/mL) of maitotoxin enhance Ca^{2+} uptake, elevate $[Ca^{2+}]_i$ and induce membrane depolarization by a mechanism that cannot be prevented by 100 nM nimodipine. It is possible that these higher concentrations of maitotoxin also involve an action at L-VDCC. In this scenario, maitotoxin by interaction with the calcium channel may alter the action of nimodipine to promote channel inactivation. Previously, we demonstrated that maitotoxin does not compete with [^{3}H]PN 200-110 binding to the dihydropyridine site of L-VDCC in membranes of GH_4C_1 cells, but does increase the amount of [^{3}H]PN 200-110 binding to intact cells [10]. This indicates that maitotoxin does not have the same binding site as nimodipine, but that maitotoxin may alter the interaction of nimodipine with L-VDCC in intact cells. Although this may be an attractive hypothesis, it still remains to be tested rigorously. None the less, this hypothesis is not supported by our findings that maitotoxin can cause Ca^{2+} uptake and membrane depolarization in the L^{tk-} cells which lack voltage-dependent calcium channels. Thus, the most feasible interpretation of these data is that the higher concentrations of maitotoxin activate an ionic mechanism in addition to L-VDCC.

Analysis of maitotoxin action by electrophysiological approaches have indicated that maitotoxin induces large inward currents [12–17]. However, these studies have found that these inward currents are voltage independent, and not voltage dependent as is predicted by pharmacologic studies. Although calcium has been suggested to be the conducted ion in some of these studies, the majority of the reports indicate that a nonselective channel, which conducts largely sodium, is activated by maitotoxin. Radioisotope studies have confirmed that the flux of several different cations is enhanced by maitotoxin treatment [9]. Additionally, this study has indicated that the flux of chloride, the counter-ion used for many of the cation studies, is also increased by maitotoxin. Our findings that reduced extracellular Cl^- increases the magnitude of depolarization induced by 0.5 ng/mL maitotoxin provides another line of evidence that Cl^- conductances participate in the membrane depolarization induced by higher concentrations of maitotoxin. More recently using patch clamp analysis of GH_4C_1 cells, we found that maitotoxin induces large inward currents that have a reversal potential close to that of chloride [17]. Because the inward currents were accentuated by iodide substitution, we have suggested that chloride may be a contributing ion to the voltage-independent depolarization induced by maitotoxin. Indeed, many sodium conductances are permeable to chloride as well [24].

The primary target for maitotoxin has been investigated and debated for several years. Different cell systems, different concentrations and even different sources of maitotoxin have been used to examine the mechanisms of maitotoxin-induced $[Ca^{2+}]_i$ increase. Several hypotheses have been postulated. In this study, we used GH_4C_1 cells to distinguish two ionic mechanisms for maitotoxin-induced Ca^{2+} entry. Direct involvement of L-VDCC is required for lower concentrations of maitotoxin. However, the large effect on $[Ca^{2+}]_i$ increase by higher concentrations of maitotoxin is nimodipine insensitive and probably requires voltage-independent mechanisms in addition to the type-L voltage-dependent calcium channel.

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