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Life Sciences 74 (2004) 2075–2083

Life Sciences

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Mercury-induced Ca^{2+} increase and cytotoxicity in renal tubular cells

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Received 11 April 2003; accepted 30 September 2003

Abstract

The effect of mercury (Hg^{2+}), a known nephrotoxicant, on intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) in Madin Darby canine kidney (MDCK) cells was explored. $[Ca^{2+}]_i$ was measured by using the Ca^{2+}-sensitive dye fura-2. Hg^{2+} increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} of 6 μM. The Ca^{2+} signal comprised a gradual increase. Removal of extracellular Ca^{2+} decreased the Hg^{2+}-induced $[Ca^{2+}]_i$ increase by 27%, suggesting that the Ca^{2+} signal was due to both extracellular Ca^{2+} influx and store Ca^{2+} release. In Ca^{2+}-free medium, the Hg^{2+}-induced $[Ca^{2+}]_i$ increase was nearly abolished by pretreatment with 1 μM thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor), and conversely, pretreatment with Hg^{2+} abolished thapsigargin-induced Ca^{2+} increase. Hg^{2+}-induced Ca^{2+} release was not altered by inhibition of phospholipase C but was potentiated by activation of protein kinase C. Overnight treatment with 1 μM Hg^{2+} did not alter cell proliferation rate and mitochondrial activity, but 10 μM Hg^{2+} killed all cells. Collectively, this study shows that Hg^{2+} induced protein kinase C-regulated $[Ca^{2+}]_i$ increases in renal tubular cells via releasing store Ca^{2+} from the endoplasmic reticulum in a manner independent of phospholipase C activity. Hg^{2+} also caused cytotoxicity at higher concentrations.
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Keywords: Ca^{2+}; MDCK cells; Mercury; Renal; Thapsigargin; Fura-2

Introduction

All forms of mercury have toxic effects in a number of organs, especially in the kidney. Within the kidney, the proximal tubule is the most vulnerable segment of the nephron to the toxic effects of

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doi:10.1016/j.lfs.2003.09.046

mercury (Zalups, 2000). The biological and toxicological activity of mercurous and mercuric ions in the kidney can be defined largely by the molecular interactions that occur at critical nucleophilic sites in and around target cells. Because of the high bonding affinity between mercury and sulfur, there is a particular interest in the interactions that occur between mercuric ions and the thiol group(s) of proteins, peptides and amino acids (Diamond and Zalups, 1998). A transient increase in the intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) is used by the cell as a key signal to trigger and regulate many pathophysiological processes, including necrosis (Berridge, 2002; Bootman et al., 2002). However, an abnormal $[Ca^{2+}]_i$ rise is cytotoxic and can lead to apoptosis, dysfunction of proteins, interference of ion flux, etc. (Annunziato et al., 2003). The Madin-Darby canine kidney (MDCK) cell line is a useful model for renal research. It has been shown that in this cell line, $[Ca^{2+}]_i$ can increase in response to the stimulation of various endogenous and exogenous agents, such as bradykinin (Jan et al., 1998b), linoleamide (Huang and Jan, 2001), organic tin compounds (Jiann et al., 2002), etc. Hg^{2+} has been shown to independently activate K^+ channels and increase $[Ca^{2+}]_i$ in MDCK cells (Jungwirth et al., 1991), but the underlying mechanism of the $[Ca^{2+}]_i$ increase is unclear. By using fura-2 as a Ca^{2+}-sensitive dye, here we show that Hg^{2+} induces concentration-dependent $[Ca^{2+}]_i$ increase both in the presence and absence of extracellular Ca^{2+} in MDCK cells. The Ca^{2+} responses are characterized, the concentration-response relationship is established, and the pathways underlying Hg^{2+}-induced Ca^{2+} release are evaluated. Furthermore, the cytotoxic effect of overnight treatment with Hg^{2+} was explored by measuring the mitochondrial activity and proliferation rate.

Materials and Methods

Cell culture

MDCK cells obtained from American Type Culture Collection were cultured in Dulbecco's modified essential medium supplemented with 10% heat-inactivated fetal bovine serum in 5% CO_2/ 95% air at 37 °C.

Solutions

Ca^{2+}-containing medium contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5; pH 7.4. Ca^{2+}-free medium contained similar components as Ca^{2+}-containing medium except that Ca^{2+} was substituted with 2 mM $MgCl_2$. Agents were dissolved in water, ethanol or dimethyl sulfoxide. Final concentrations of organic solvents in the experimental solution were less than 0.1% which did not alter basal $[Ca^{2+}]_i$ (n = 3; not shown). $HgCl_2$ was dissolved in water as a 10 mM stock and was diluted to the final concentrations before assays.

$[Ca^{2+}]_i$ measurements

Trypsinized cells (10^6/ml) were allowed to recover in culture medium for 1 hr before loading with 2 μM fura-2/acetoxy methyl for 30 min at 25 °C in the same medium. The cells were washed and resuspended in Ca^{2+}-containing medium. Fura-2 fluorescence measurements were performed in a water-

jacketed cuvette (25 °C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Kyoto, Japan) by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a Kd of 155 nM (Grynkiewicz et al., 1985).

Colorimetric assay

Cytotoxicity tests were carried out using WST-1, a fluorescent cell proliferation reagent. The assay is based on cleavage of the tetrazolium salt WST-1 by active mitochondria to produce a soluble colored formazan salt (Ishiyama et al., 1996). Since the conversion is operated only by viable cells, it directly correlates with the cell number. The cells were plated at 1×10^4 in 96-well microtiter plates. Twenty-four hours after plating, at 70% confluence the growth medium was removed and replaced with the test solutions (100 µl). After 16-hour exposure the reaction medium was removed, the cells were washed twice with culture medium, then 100 µl culture medium and 10 µl WST-1 were added to each well. The cells were incubated for 2 hours at 37°C in a humidified atmosphere with 5% CO_2, then the microplate was thoroughly shaken for 1 min and the absorbance was measured at 450 nm using a microtiter reader (model MRX II DYNEX Technologies, Chantily, VA, USA).

Chemicals

The agents for cell culture were from Gibco (Gaithersburg, MD, USA). Fura-2/acetoxy methyl was from Molecular Probes (Eugene, OR, USA). U73122 (1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) and U73343 (1-(6-((17b-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)-hexyl)-2,5-pyrrolidine-dione) were from Biomol (Plymouth Meeting, PA, USA). $HgCl_2$ and other agents were from Sigma (St. Louis, MO, USA).

Statistics

All data are presented as the means ± S.E.M. of five separate experiments. Statistical comparisons were determined by using Student's *t* test, and significance was accepted when $P < 0.05$.

Results

Effect of Hg^{2+} on $[Ca^{2+}]_i$

Fig. 1A shows that in Ca^{2+}-containing medium, basal $[Ca^{2+}]_i$ was 50 ± 2 nM (n = 5). Hg^{2+} (5–20 µM) caused a gradual $[Ca^{2+}]_i$ increase, which lasted for, at least, 370 s after the addition of Hg^{2+}; e.g. Hg^{2+} (20 µM)-induced $[Ca^{2+}]_i$ increase attained to 151 ± 3 nM (n = 5) over baseline at the time point of 400 s. The signal sustained and did not decay. The effect of Hg^{2+} was concentration-dependent, and saturated at 20 µM of the agent with an EC_{50} value of 6 µM (Fig. 1B).

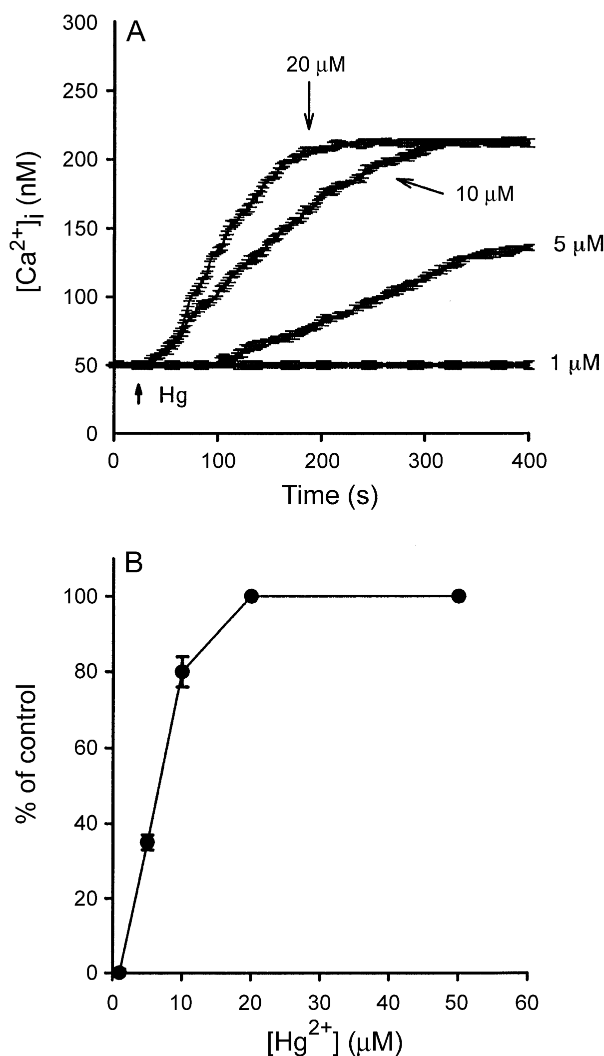

Fig. 1. Effects of Hg^{2+} on $[Ca^{2+}]_i$. (A) Concentration of the reagent was 1–20 μM. The control response was the same as the one induced by 1 μM Hg^{2+}. The experiments were performed in Ca^{2+}-containing medium. Hg^{2+} was added at 30 s and was present throughout the measurement of 400 s. (B) A concentration-response curve of Hg^{2+}-induced Ca^{2+} signals. Y axis is the percentage of control which is the net area under the curve (baseline subtracted) of the $[Ca^{2+}]_i$ increase induced by 20 Hg^{2+}. Data are mean ± S.E.M. of five experiments.

Effect of removal of extracellular Ca^{2+} on Hg^{2+}-induced $[Ca^{2+}]_i$ increases

To examine whether/how influx of extracellular Ca^{2+} and/or mobilization of Ca^{2+} from the intracellular store site(s) may contribute to Hg^{2+}-induced $[Ca^{2+}]_i$ increases, the effect of Hg^{2+} on $[Ca^{2+}]_i$ was measured in the absence of extracellular Ca^{2+}. Fig. 2A shows that the $[Ca^{2+}]_i$ increase caused by 20 μM Hg^{2+} was attenuated, with no change in basal $[Ca^{2+}]_i$ (51 ± 1 nM, n = 5). Hg^{2+}

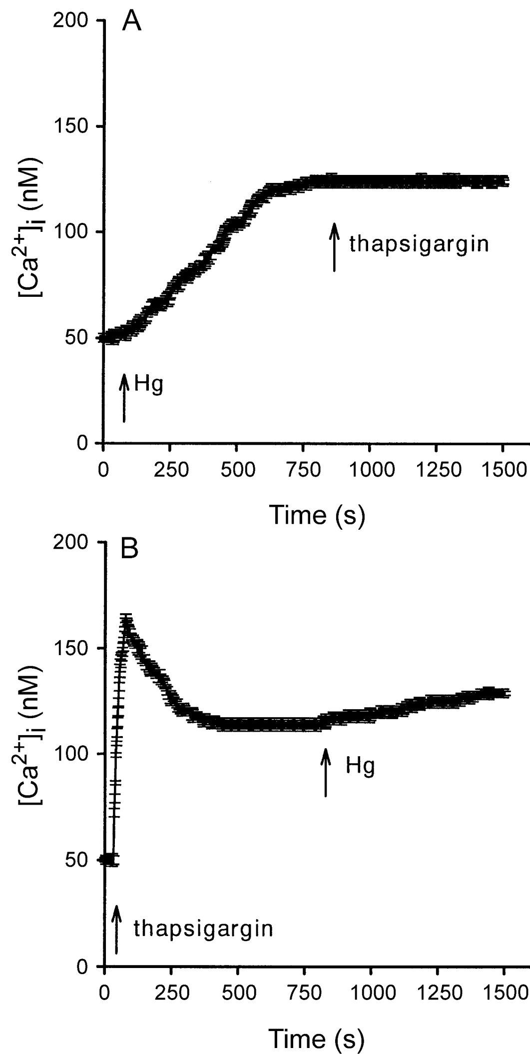

Fig. 2. Intracellular sources of Hg^{2+}-induced $[Ca^{2+}]_i$ increases. (A) and (B), all experiments were performed in Ca^{2+}-free medium. Reagents were applied at the time indicated by arrows. The concentration of reagents was 20 μM for Hg^{2+} and 1 μM for thapsigargin. Data are means ± S.E.M. of five experiments.

increased $[Ca^{2+}]_i$ by 110 ± 2 nM at the time point of 400 s, which was 27% smaller than that observed in Ca^{2+}-containing medium at the same time point. This suggests that Hg^{2+} induced both extracellular Ca^{2+} influx and intracellular Ca^{2+} release.

Mobilization by Hg^{2+} of intracellular Ca^{2+} from the endoplasmic reticulum

We examined whether Hg^{2+}-induced $[Ca^{2+}]_i$ increases may involve the mobilization of intracellular Ca^{2+} sequestered within the endoplasmic reticulum, a major Ca^{2+} store in MDCK cells (Jan et al.,

1998a; Huang and Jan, 2001; Jiann et al., 2002). Fig. 2A shows that in Ca^{2+}-free medium, 1 μM thapsigargin (1 μM), an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase (Thastrup et al., 1990), failed to cause a $[Ca^{2+}]_i$ increase (n = 5). In contrast, Fig. 2B shows that thapsigargin (1 μM) increased $[Ca^{2+}]_i$ by 115 ± 3 nM (n = 5). Furthermore, addition of 20 μM Hg^{2+} after thapsigargin treatment for 700 s only induced a negligible $[Ca^{2+}]_i$ increase (n = 5).

Lack of involvement of phospholipase C in Hg^{2+}-induced Ca^{2+} release

The role of phospholipase C-inositol 1,4,5-trisphosphate (IP_3) pathway in Hg^{2+}-induced intracellular Ca^{2+} mobilization from the endoplasmic reticulum was investigated. Fig. 3A shows that 10 μM ATP, an agonist for P2Y type ATP receptors that mobilizes intracellular Ca^{2+} from the endoplasmic reticulum via activation of phospholipase C in MDCK cells (Jan et al., 1998a), caused an instantaneous monophasic $[Ca^{2+}]_i$ increase (121 ± 2 nM, n = 5) in Ca^{2+}-free medium. Fig. 3B, however, shows that pretreatment with 2 μM U73122, an inhibitor of phospholipase C (Thompson et al., 1991), abolished ATP-induced $[Ca^{2+}]_i$ increases; in contrast, 10 μM U73343, a biologically inactive analogue of U73122 (Thompson et al., 1991), failed to prevent ATP-induced $[Ca^{2+}]_i$ increases (data not shown; n = 5). Even in the presence of 2 μM U73122, 20 μM Hg^{2+} caused a significant $[Ca^{2+}]_i$ increase which was indistinguishable from the control Hg^{2+} response (Fig. 2A).

Involvement of protein kinase C in Hg^{2+}-induced $[Ca^{2+}]_i$ increases

In MDCK, it has been shown that protein kinase C may play a role in a Ca^{2+} signal (Jan et al., 1998b). The data in Fig. 4 show that pretreatment with phorbol myristate acetate (PMA; 10 nM) to

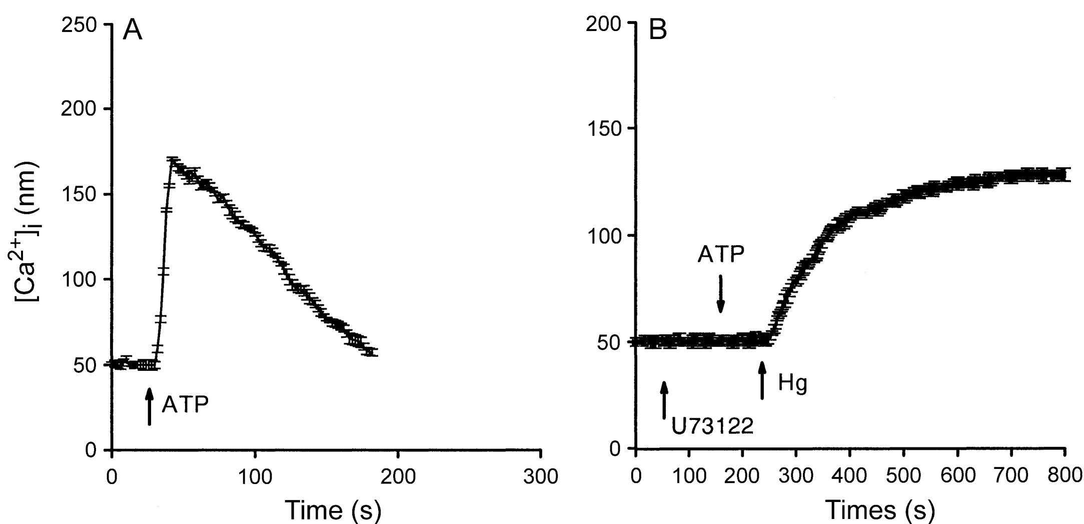

Fig. 3. Effect of U73122 on Hg^{2+}-induced $[Ca^{2+}]_i$ increases. (A) ATP (10 μM) was added at 25 s. (B) U73122 (2 μM), ATP (10 μM), Hg^{2+} (20 μM) were added at 50, 150 and 230 s, respectively. All experiments were performed in Ca^{2+}-free medium. Data are means ± S.E.M. of five experiments.

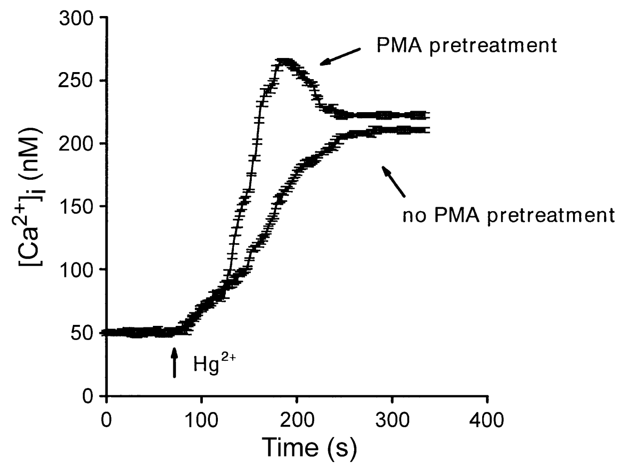

Fig. 4. Effects of phorbol myristate acetate (PMA) on Hg^{2+}-induced $[Ca^{2+}]_i$ increases. The experiments were performed in Ca^{2+}-containing medium. Hg^{2+} (20 μM) was added at 90 s. In the upper trace, PMA (10 nM) was added 60 s prior to Hg^{2+}. In the lower trace, no PMA was added prior to Hg^{2+}. Data are the means ± S.E.M. of five experiments.

activate protein kinase C caused a significant enhancement in 20 μM Hg^{2+}-induced $[Ca^{2+}]_i$ increases in Ca^{2+}-containing medium. The net maximum value was increased by 40% (211 ± 2 nM vs. 150 ± 3 nM; n = 5; $P < 0.05$).

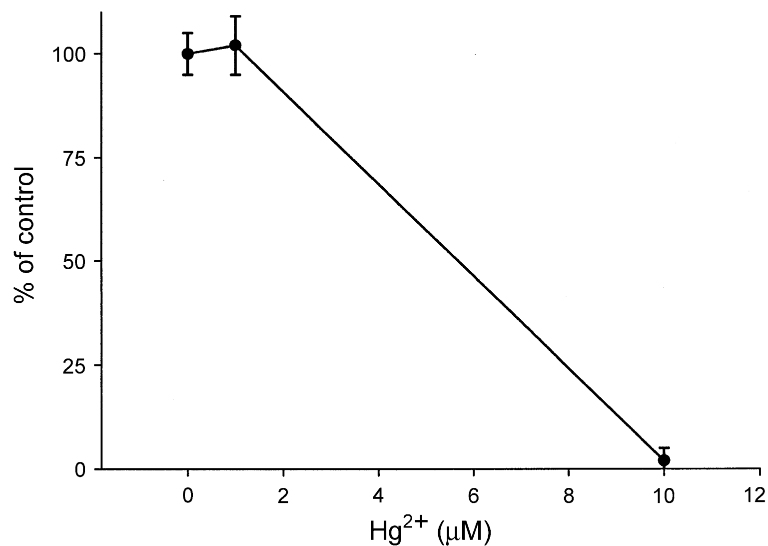

Fig. 5. Cytotoxic effect of Hg^{2+}exposure on renal tubular cells. The cell viability assay is described in Materials and methods Hg^{2+} (0,1, and 10μM) was added to cells for 16 hours. Data are expressed as the percentage of control (no Hg^{2+} was present). Control had 10, 242 ± 3 cells/well before experiments, and had 12, 121 ± 45 cells/well after incubation for 16 hours. Data are means ± S.E.M. of five experiments in six replicates (wells).

Cytotoxic effect of Hg^{2+} on renal tubular cells

It is well established that unregulated, prolonged $[Ca^{2+}]_i$ increases may lead to cytotoxicity (Berridge, 2002), thus experiments were performed to examine the effect of overnight incubation with Hg^{2+} on the proliferation of MDCK cells. In control groups, the cell number per well increased by 18.3 $\pm$ 2.1% (from 10,242 $\pm$ 3 to 12,121 $\pm$ 45; n = 5; six replicates in each experiment; $P < 0.05$). In the presence of 1 μM Hg^{2+}, the cell number did not change. However, at a concentration of 10 μM, Hg^{2+} treatment reduced the cell number to 2 $\pm$ 3% of control (n = 5; $P < 0.05$) Fig. 5.

Discussion

Cytotoxicity of Hg^{2+} on renal tubular cells has been reported previously. In MDCK cells, Hg^{2+} (~ 10 μM) was shown to inhibit gap junction function (Aleo et al., 2002), mitochondrial dehydrogenase activity, thymidine incorporation and protein content (Bohets et al., 1995). Our data support these observations by showing that overnight incubation with 10 μM Hg^{2+} caused cell death. Although the mechanisms underlying the Hg^{2+}-induced cytotoxicity are not completely clear, an increase in $[Ca^{2+}]_i$ may play a pivotal role. The present study shows that Hg^{2+} causes a concentration-dependent $[Ca^{2+}]_i$ increase. The data show that Hg^{2+} increases $[Ca^{2+}]_i$ by causing both store Ca^{2+} release and extracellular Ca^{2+} influx because the response was partly reduced by removal of extracellular Ca^{2+}. The dominant Ca^{2+} influx pathway in MDCK cells has been shown to be via store-operated Ca^{2+} entry (Jan et al., 1998a), a process triggered by depletion of the endoplasmic reticulum Ca^{2+} (Putney, 1985). How Hg^{2+} causes Ca^{2+} influx in a non-excitable cell line such as MDCK cells is unclear. In cerebellar granule cells, mercury is shown to alter Ca^{2+} homeostasis through nifedipine- and omega-conotoxin-MVIIC-sensitive pathways, suggesting that L-, N-, and/or Q-type Ca^{2+} channels may play a role in mercury's mode of action or entry (Marty and Atchison, 1997). In T cells, Hg^{2+} was thought to evoked Ca^{2+} influx through L-type Ca^{2+} channels (Badou et al., 1997).

Regarding the intracellular Ca^{2+} stores of the Hg^{2+} response, the thapsigargin-sensitive endoplasmic reticulum store, the dominant Ca^{2+} store in MDCK cells (Jan et al., 1998b), appears to play a major role because Hg^{2+} completely depleted the endoplasmic reticulum Ca^{2+} store, and vice versa, thapsigargin treatment nearly abolished Hg^{2+}-induced Ca^{2+} release. Furthermore, the Hg^{2+}-induced Ca^{2+} release does not require a preceding elevation in cytosolic IP_3 levels because this release was not changed by inhibition of phospholipase C. The IP_3-independent component(s) of the Ca^{2+} releasing event is unknown, but may be related to inhibition of Ca^{2+} pump or permeabilization of the endoplasmic reticulum membranes.

It has been shown that protein kinase C activation is involved in the mediation of Hg^{2+}-induced Ca^{2+} influx through L-type Ca^{2+} channels in T cells (Badou, 1997). Protein kinase C has also been shown to potentiate the $[Ca^{2+}]_i$ increases induced by bradykinin in MDCK cells (Jan et al., 1998b). These lines of evidence prompted us to explore the role of protein kinase C in Hg^{2+}-induced $[Ca^{2+}]_i$ increases in MDCK cells, and found that the Ca^{2+} signal was enhanced by activation of protein kinase C. This suggests that Hg^{2+} induces $[Ca^{2+}]_i$ increases in MDCK cells via regulated mechanisms. Together, this study shows that Hg^{2+} induced protein kinase C-regulated $[Ca^{2+}]_i$ increases in renal tubular cells via releasing store Ca^{2+} from the endoplasmic reticulum in a manner independent of phospholipase C activity. Hg^{2+} also induced cytotoxicity at higher concentrations.

Acknowledgements

This work was supported by grants from Veterans General Hospital-Kaohsiung (VGHKS91-48 and VGHKS92-36 to J.-H. Yeh and VGHKS92G-11 to C.R. Jan).

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