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Characteristics of lysophosphatidylcholine-induced Ca^{2+} response in human neuroblastoma SH-SY5Y cells

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Abstract

Lysophosphatidylcholine (LPC) is an important bioactive lipid. In the nervous system, elevated levels of LPC have been shown to produce demyelination. In the present study, we examined the effect of exogenous LPC on intracellular Ca^{2+} mobilization in human neuroblastoma SH-SY5Y cells. In Ca^{2+}-containing medium, introduction of LPC induced a steady rise in cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) in a dose-dependent manner, and this rise was provoked by LPC itself, not by its hydrolysis product produced by lysophospholipase. The increase in $[Ca^{2+}]_i$ was reduced by 36% by removal of extracellular Ca^{2+}, while preincubation of the cells with verapamil, an L-type Ca^{2+} channel blocker, inhibited the response by 23%, part of the Ca^{2+} influx. Conversely, Ni^{2+}, which inhibits the Na^+–Ca^{2+} exchanger, or Na^+-deprivation did not affect LPC-induced Ca^{2+} influx. In Ca^{2+}-free medium, depletion of Ca^{2+} stores in the endoplasmic reticulum (ER) by thapsigargin, an ER Ca^{2+}-ATPase inhibitor, abolished the Ca^{2+} increase. Moreover, LPC-induced $[Ca^{2+}]_i$ increase was fully blocked by ruthenium red and procaine, inhibitors of ryanodine receptor (RyR), but was not affected by 2-aminoethoxydiphenyl borate, an inhibitor of inositol triphosphate receptor, or by pertussis toxin, a $G_{i/o}$ protein inhibitor. Combined treatment with verapamil plus thapsigargin markedly inhibited but did not abolish the LPC-induced Ca^{2+} response. These findings indicate that LPC-induced $[Ca^{2+}]_i$ increase depends on both external Ca^{2+} influx and Ca^{2+} release from ER Ca^{2+} stores, in which L-type Ca^{2+} channels and RyRs may be involved. However, in digitonin-permeabilized SH-SY5Y cells, LPC could not induce any $[Ca^{2+}]_i$ increase in Ca^{2+}-free medium, suggesting that LPC may act indirectly on RyRs of ER.

Keywords: Lysophosphatidylcholine; Demyelination; Endoplasmic reticulum Ca^{2+} store; Calcium influx; Ryanodine receptors; Human neuroblastoma SH-SY5Y cells

Introduction

Lysophosphatidylcholine (LPC), also called lysolecithin, is an important lipid molecule in mammalian tissues. Elevated LPC levels are associated with a host of disorders such as atherosclerosis, myocardial ischemia, and inflammatory diseases (Vidaver et al., 1985; Quinn et al., 1988; Sedlis et al., 1993). Although LPC is also a major lipid constituent that is associated with several types of neuroactivity in nervous system (Wang et al., 1997), it can also induce neuronal sheath demyelination, together with a variable degree of axonal degeneration (Hall, 1972; Jean et al., 2002), which is a common pathological characteristic of neurodegenerative diseases (Kostrzewa and Segura-Aguilar, 2003; Iqbal et al., 2003).

Neuropathy target esterase (NTE) is a neuronal membrane protein in vertebrates, and was originally identified as the primary action site of those organophosphorus compounds (OP) that induce a delayed neuropathy characterized by demyelination and degeneration of long nerve axons (Johnson, 1969; Bouldin and Cavanagh, 1979). Recently, NTE was characterized as a novel lysophospholipase (NTE-LysoPLA) that targeted LPC as its physiological substrate (Quistad et al., 2003). Moreover, elevated levels of LPC have been detected in spinal cord but not in brain following OP treatment (Quistad and Casida, 2004). Because the adverse effects of LPC, i.e., demyelination and axonal lesion, are similar to the pathological

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changes of OP-induced delayed neuropathy (OPIDN), it was supposed that inhibition of NTE-LysoPLA activity may produce a localized increase in LPC levels and thus induce a cell signalling cascade, which leads to demyelination and, ultimately, neurodegradation of nerve axons.

An increase in cytosolic Ca^{2+} level ($[Ca^{2+}]_i$) has been implicated in progressive events that lead to delayed neuropathies resulting from OPs (Abou-Donia and Lapadula, 1990; Choudhary and Gill, 2001). Increased $[Ca^{2+}]_i$ may cause the breakdown of cytoskeletal elements in various neurodegenerative states (Schlaepfer and Hasler, 1979; Schlaepfer, 1987). Moreover, the calcium ionophore ionomycin or calcium itself has been observed to induce central demyelination and axonal degeneration in vivo and the appearance and progression of the lesion was strikingly similar to that seen after intraneural injection of LPC (Smith and Hall, 1988, 1994). Furthermore, as a bioactive lipid, LPC has been shown to enhance intracellular Ca^{2+} levels in many cell types (Okajima et al., 1998; Yu et al., 1998). As such, we postulated that LPC-induced demyelination and axonal degeneration, which might be involved in the pathogenesis of OPIDN, may result from LPC-induced Ca^{2+} overload.

The effect of LPC-induced Ca^{2+} overload has been widely investigated on those cell types involved in atherosclerosis and inflammatory diseases, such as endothelial cells, monocytes, macrophages, and cardiac myocytes (Okajima et al., 1998; Yu et al., 1998). However, the action(s) of LPC on neurocytes remain poorly characterized. In the present study, we examined the effect and possible mechanisms of LPC on the changes in intracellular Ca^{2+} levels in SH-SY5Y neuroblastoma cell line, which has been widely used in the investigation of neurotoxicity.

Materials and methods

Materials

SH-SY5Y cells were obtained from the Cell Center of Chinese Academy of Medical Sciences (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (Grand Island, NY, U.S.A.). Heat-inactivated fetal bovine serum (FBS) was purchased from the Chinese Academy of Medical Sciences (Beijing, China) and trypsin was purchased from Amresco (Solon, OH, U.S.A.). Fura-2/AM, L-a-Lysophosphatidylcholine (primary palmitate and stearate esters) (LPC), *sn*-glycero-3-phosphocholine (GPC), verapamil, ruthenium red, pertussis toxin (PTX), 2-aminoethoxydiphenyl borate (2-APB), thapsigargin, digitonin, and $NiCl_2$ were purchased from Sigma (St. Louis, MO, USA).

Cell culture

SH-SY5Y neuroblastoma cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were passaged once a week and used in confluent culture in all the subsequent experiments.

Intracellular Ca^{2+} measurements

$[Ca^{2+}]_i$ was determined using the Ca^{2+}-sensitive fluorescent indicator Fura-2/AM. Cells were washed, resuspended in HEPES-buffered medium (140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml glucose and 20 mM HEPES, pH 7.4) at 1×10^7 cells/ml, and then incubated with 2.5 μM Fura-2/AM for 30 min at 37 °C. Cells were washed twice, and resuspended in HEPES buffer at 1×10^6 cells/ml. Fluorescence intensity was monitored at 510 nm (5 nm slit) with alternated excitation at 340 nm and 380 nm (5 nm slit) using a dual wavelength fluorescence spectrophotometer (Model F4500; Hitachi, Tokyo, Japan).

$[Ca^{2+}]_i$ was calculated using FL Solutions 2.0 Intracellular Cation Scan software based on the formula described by Grynkiewicz et al. (1985), assuming a dissociation constant kd of 224 nM. Maximal and minimal fluorescence values were obtained at the end of the experiment by sequential addition of 0.1% Triton X-100 and 5 mM EGTA.

In some experiments, Fura-2 loaded cells were washed and suspended in normally Ca^{2+}-free medium which contained the same components as HEPES-buffered medium except that $CaCl_2$ was omitted. In Ca^{2+} restoration experiments, Ca^{2+} was initially absent from the medium, and was restored to 1 mM after 250 s of LPC stimulation. In some other experiments, Fura-2 loaded cells were permeabilized with 5 μM digitonin in Ca^{2+}-free medium for 5 min at 25 °C, and the cells were then washed twice and resuspended in Ca^{2+}-free medium without digitonin.

Drug treatments

Where indicated, cells were pretreated with inhibitors of Ca^{2+} signalling proteins prior to their challenge with 10 μM LPC. In particular, where indicated, cells were preincubated with verapamil, procaine, or 2-APB for 15 min, or with thapsigargin for 10 min, or with ruthenium red, or Ni^{2+} for 5 min, followed by stimulation with 10 μM LPC. Where indicated, cells were incubated with PTX supplemented into the culture medium for 24 h prior to their use in experiments.

Statistical analysis

Comparisons between different groups were performed using Student's two-tailed unpaired *t*-test or a one-way analysis of variance (Dunnett's test). $P<0.05$ was considered to be a significant difference. All values are given as means ± SD.

Results

Ca^{2+} signals induced by LPC

We first examined the effect of LPC on Ca^{2+} mobilization in SH-SY5Y neuroblastoma cells. As shown in Fig. 1A, introduction of LPC induced a gradual increase in $[Ca^{2+}]_i$ incubated in Ca^{2+}-containing medium in a dose-dependent manner. In particular, the rise in $[Ca^{2+}]_i$ was slow, eventually reaching a

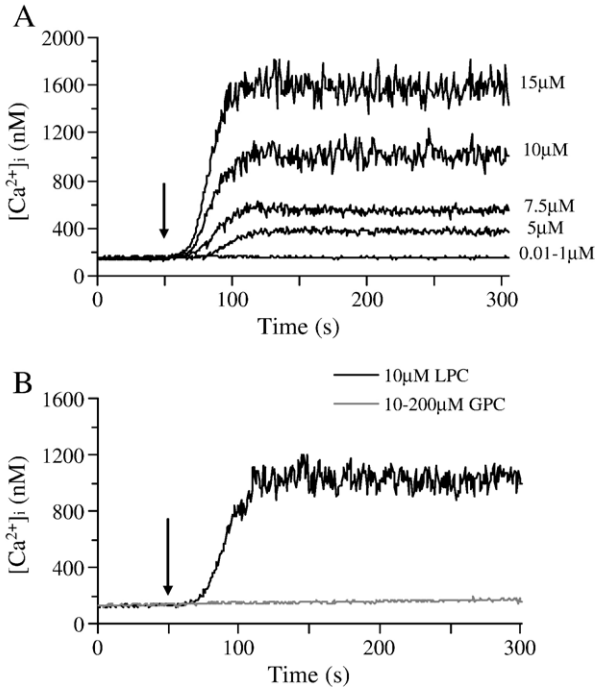

Fig. 1. Ca^{2+} responses to different concentrations of LPC and GPC in SH-SY5Y neuroblastoma cells. Fura-2 loaded cells were stimulated by the addition of LPC or GPC, a hydrolysis product of LPC, in Ca^{2+}-containing medium. Arrows indicate the time of LPC or GPC addition. Traces are representative of at least 4 experiments. A: Typical $[Ca^{2+}]_i$ responses of SH-SY5Y cells to different concentrations of LPC. Numbers next to the traces indicate concentrations of LPC. B: Comparison of Ca^{2+} responses to LPC (black) and GPC (grey). GPC concentrations range from 10 to 200 μM.

large and sustained plateau. As LPC can be hydrolyzed by NTE-LysoPLA, we speculated that the Ca^{2+} increase by LPC might be provoked by the hydrolysis product of LPC. However, experiments with GPC, a hydrolysis product of LPC showed that up to 200 μM could not cause any Ca^{2+} increase under the same experimental conditions (Fig. 1B). The concentration of LPC above 10 μÌ is sufficient to induce large Ca^{2+} responses, which are potent enough to stimulate downstream Ca^{2+}-sensitive processes. Moreover, cell viability measurements using trypan blue exclusion showed that while 15 μM LPC caused about 30% of the cells to stain with the dye, whereas cells exposed to 10 μM LPC showed no significant dye infiltration under the experimental conditions (data not shown). Hence, 10 μM LPC was used in all the subsequent studies.

Sources of Ca^{2+} signals mediated by LPC

We then measured the effect of 10 μM LPC on Ca^{2+} response in the presence or absence of extracellular Ca^{2+} ($Ca^{2+}{}_e$). The amplitude of $[Ca^{2+}]_i$ increase evoked by LPC was decreased by 36% in the absence of $Ca^{2+}{}_e$, compared with that in the presence of $Ca^{2+}{}_e$ (Fig. 2A, B). Similar results were obtained from Ca^{2+} restoration experiments where a further increase in $[Ca^{2+}]_i$ was observed when 1 mM Ca^{2+} was added back to the normally Ca^{2+}-free buffer after LPC stimulation (Fig. 2C); hence this additional increase in $[Ca^{2+}]_i$ is attributed solely to Ca^{2+} entry from external medium.

Inhibitory effect of Ca^{2+} channel blocker on LPC-induced $[Ca^{2+}]_i$ increase

To identify the source of Ca^{2+} influx induced by LPC, we investigated the effect of verapamil, an L-type Ca^{2+} channel blocker, on LPC-induced $[Ca^{2+}]_i$ elevation. Pretreatment of cells with 20 μM verapamil inhibited the LPC response by 23% compared to the absence of verapamil in Ca^{2+}-containing medium (Fig. 3A, B). No further inhibitory effect was observed with higher concentrations of verapamil.

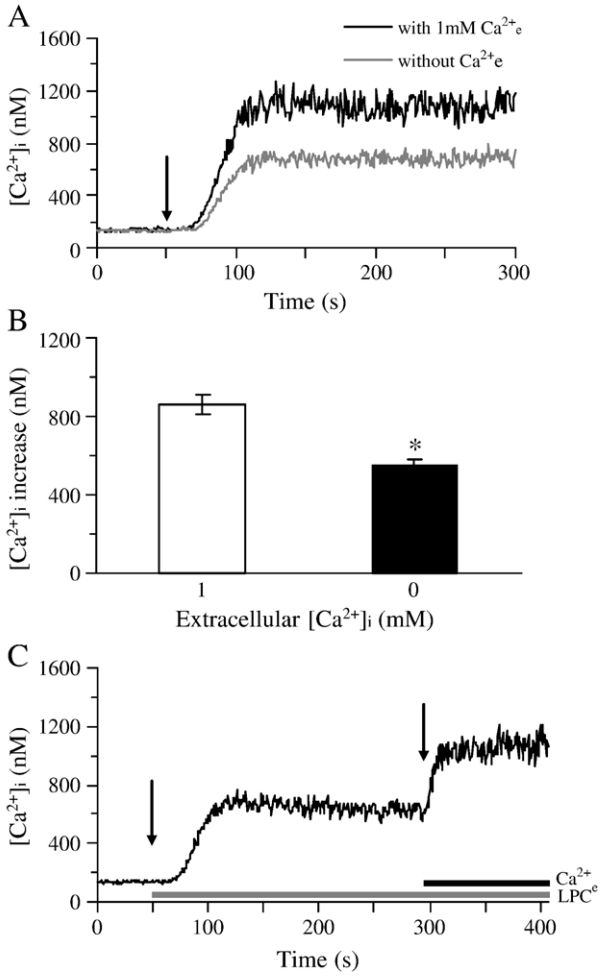

Fig. 2. Sources of LPC-mediated Ca^{2+} signals in SH-SY5Y human neuroblastoma cells. Fura-2 loaded cells were stimulated with 10 μM LPC in the presence or absence of extracellular Ca^{2+} ($Ca^{2+}{}_e$) in medium. A: Typical traces of $[Ca^{2+}]_i$ responses in the presence (black) or absence (grey) of 1 mM $Ca^{2+}{}_e$. Arrow indicates the time of LPC addition. Traces are representative of at least 4 experiments. B: Average $[Ca^{2+}]_i$ increase above basal level in the presence (□) or absence (■) of $Ca^{2+}{}_e$. Data are means ± SD from at least 4 separate experiments. *$P<0.05$. C: Typical traces for $[Ca^{2+}]_i$ changes during the course of Ca^{2+} restoration. Ca^{2+} was initially absent from the medium, and was restored to 1 mM as indicated (second arrow) 250 s after LPC stimulation (first arrow). Solid bars indicate the presence of LPC (grey) and $Ca^{2+}{}_e$ (black). Traces are representative of at least 3 experiments.

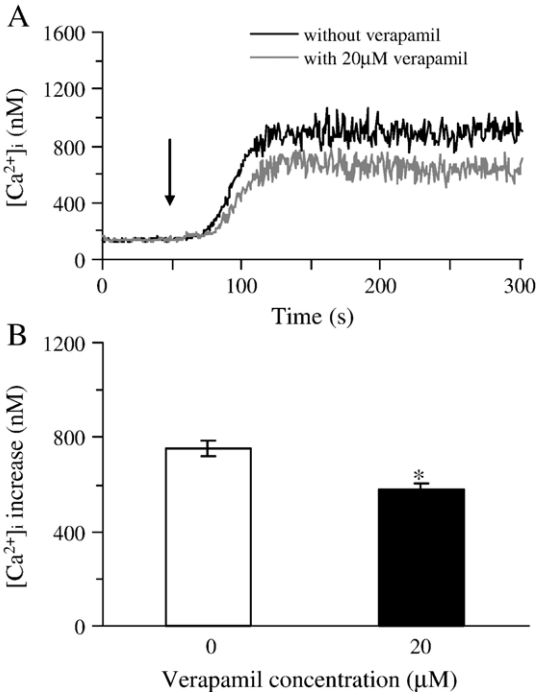

Fig. 3. Effect of verapamil on LPC-induced $[Ca^{2+}]_i$ increase. Fura-2 loaded cells were pretreated with vehicle (control) or with 20 μM verapamil for 15 min, then stimulated with 10 μM LPC in Ca^{2+}-containing medium. A: Typical Ca^{2+} traces by LPC in the absence (black) or presence (grey) of verapamil. Arrow indicates the time of LPC addition. Traces are representative of at least 4 experiments. B: Average $[Ca^{2+}]_i$ increase above basal level in the absence (□) or presence (■) of verapamil. Data are means±SD from at least 4 separate experiments. *$P<0.05$.

Dependence of endoplasmic reticulum (ER) in LPC-induced Ca^{2+} rise

To explore role of ER in LPC-induced Ca^{2+} rise in SH-SY5Y neuroblastoma cells, we measured the LPC-induced $[Ca^{2+}]_i$ increase in Ca^{2+}-free medium. As shown in Fig. 4, the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin (5 μM), which depletes Ca^{2+} stores in ER, abolished the LPC-induced $[Ca^{2+}]_i$ increase. This indicated that mobilization of intracellular Ca^{2+} stores from ER was involved in LPC-induced $[Ca^{2+}]_i$ increase in neuroblastoma SH-SY5Y cells.

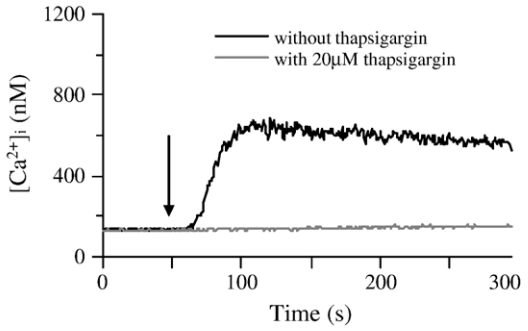

Fig. 4. Role of ER in LPC-induced $[Ca^{2+}]_i$ increase. Changes in $[Ca^{2+}]_i$ were monitored after pretreatment with thapsigargin, followed by addition of 10 μM LPC in Ca^{2+}-free medium. Typical $[Ca^{2+}]_i$ response to LPC after pretreatment of cells with vehicle (black) or with 5 μM thapsigargin (grey) for 10 min. Arrow indicates the time of LPC addition. Traces are representative of at least 3 experiments.

Independence of inositol trisphosphate receptor (IP3R) activation

We also investigated the possible involvement of $G_{i/o}/G_{q/11}$ G protein-phospholipase C-IP_3 receptor pathway in LPC-induced $[Ca^{2+}]_i$ increase in neuroblastoma cells. In order to exclude the influence of $Ca^{2+}{}_e$, we conducted the experiments in Ca^{2+}-free medium. As shown in Fig. 5, pretreatment of cells with the cell-permeant IP_3R inhibitor 2-APB for 15 min had no effect on LPC-induced Ca^{2+} response (Fig. 5A). Moreover, 24-hour pretreatment of cells with PTX, a known inhibitor of $G_{i/o}$ proteins, did not have any inhibitory effect on the Ca^{2+} response (Fig. 5B).

Role of ryanodine receptor (RyR) in LPC-induced Ca^{2+} response

We next tested the role of RyRs, another class of intracellular Ca^{2+} releasing channels, in LPC-induced Ca^{2+} response in SH-SY5Y neuroblastoma cells. As before, experiments were conducted in Ca^{2+}-free medium to minimize the Ca^{2+} entry interference. Ruthenium red, a potent RyR inhibitor, inhibited the LPC-induced Ca^{2+} rise in a dose-dependent manner. Pretreatment of cells with 30 μM ruthenium red for 5 min completely blocked LPC-induced Ca^{2+} rise (Fig. 6A).

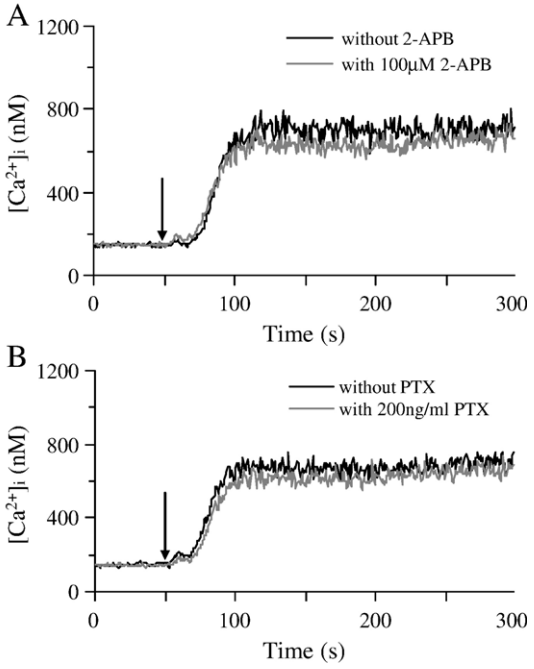

Fig. 5. Lack of effects of 2-APB and PTX on LPC-induced $[Ca^{2+}]_i$ increase. Changes in $[Ca^{2+}]_i$ were monitored after pretreatment with appropriate inhibitors, followed by addition of 10 μM LPC in Ca^{2+}-free medium. Arrows indicate the time of LPC addition. Data are representative of at least 3 experiments. A: Typical Ca^{2+} traces after pretreatment of cells with vehicle (black) or with 100 μM 2-APB (grey) for 15 min. B: Typical Ca^{2+} traces in the absence (black) or presence (grey) of PTX. PTX was added to culture medium before experiments with 100 ng/ml for 24 h.

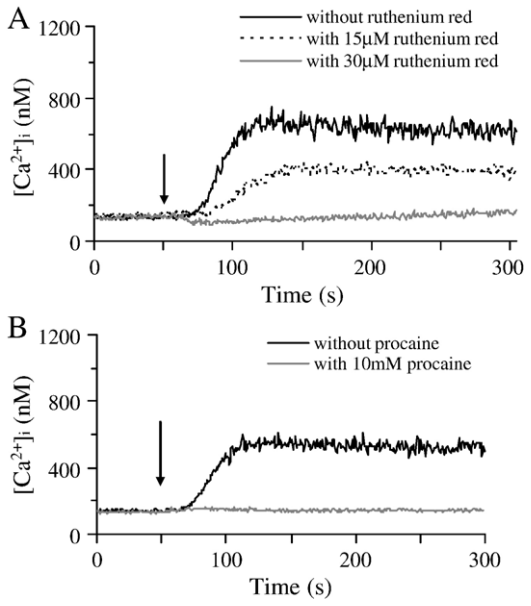

Fig. 6. Role of RyRs on LPC-induced Ca^{2+} response. Fura-2 loaded cells were preincubated with appropriate inhibitors and then stimulated with 10 μM LPC in Ca^{2+}-free medium. Arrows indicate the time of LPC addition. Traces are representative of at least 3 experiments. A: Typical $[Ca^{2+}]_i$ changes after pretreatment with vehicle (black line) or with 15 μM (grey dots) or 30 μM ruthenium red (grey line) for 5 min. B: Typical $[Ca^{2+}]_i$ changes after pretreatment with vehicle (black) or with 10 mM procaine (grey) for 15 min.

Moreover, as shown in Fig. 6B, in cells pretreated for 15 min with 10 mM procaine, another well-known inhibitor for RyR, there was also strong inhibition of LPC-induced Ca^{2+} rise.

Indirect action of LPC on the RyR

In order to determine whether LPC directly stimulates the RyRs, we measured Ca^{2+} response to LPC in digitonin-permeabilized cells in Ca^{2+}-free medium, in which the chemical was allowed to access to the intracellular Ca^{2+}-release channel to interact directly with the receptor. As shown in Fig. 7, LPC did not induce any $[Ca^{2+}]_i$ increase in the digitonin-permeabilized cells, suggesting that LPC did not directly act on RyRs to induce the Ca^{2+} release.

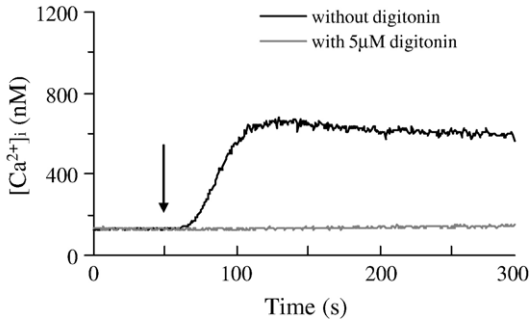

Fig. 7. Indirect action of LPC on RyR. Cells were permeabilized by incubating with 5 μM digitonin in Ca^{2+}-free medium for 5 min at 25 °C. Fura-2 loaded cells were stimulated with 10 μM LPC in the absence of extracellular Ca^{2+} in the medium. Typical Ca^{2+} response to LPC in intact cells (black) or permeabilized cells (grey). Arrow indicates the time of LPC addition. Traces are representative of at least 3 experiments.

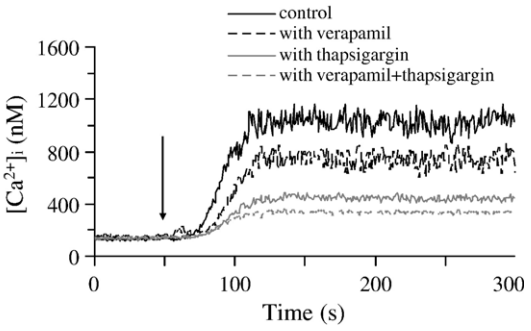

Fig. 8. Combined inhibition of LPC-induced Ca^{2+} increase by verapamil and thapsigargin. $[Ca^{2+}]_i$ changes were monitored with the addition of 10 μM LPC after appropriate inhibitors pretreatments in Ca^{2+}-containing medium. Where indicated, Fura-2 loaded cells were preincubated with vehicle (black) or 20 μM verapamil (black dash) for 15 min, or 5 μM thapsigargin (grey) for 10 min or 20 μM verapamil plus 5 μM thapsigargin (grey dash). Arrow indicates the time of LPC addition. Traces are representative of at least 3 experiments.

Possible involvement of Ca^{2+} extrusion in LPC-induced $[Ca^{2+}]_i$ increase

We examined the inhibitory effect of LPC on Ca^{2+} extrusion by pretreatment of the cells with Ca^{2+} blocker and SERCA inhibitor in Ca^{2+}-containing medium. As shown in Fig. 8, LPC-

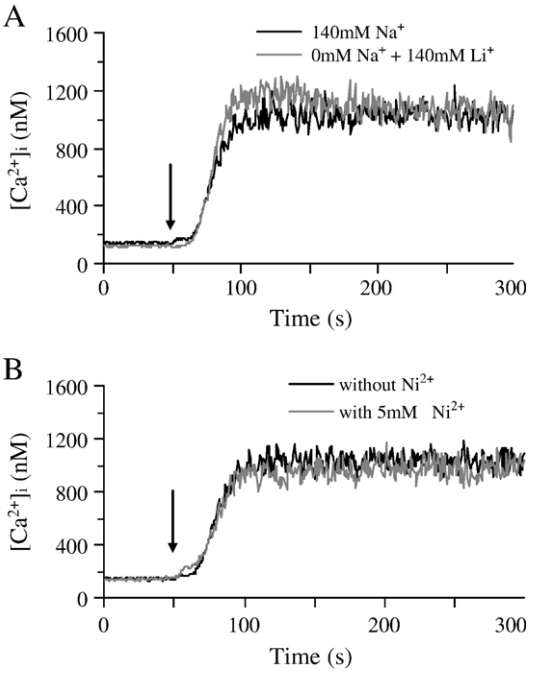

Fig. 9. Lack of involvement of Na^+–Ca^{2+} exchanger. $[Ca^{2+}]_i$ changes were monitored after addition of 10 μM LPC in Ca^{2+}-containing medium. Arrows indicate the time of LPC addition. Traces are representative of at least 3 experiments. A: Typical $[Ca^{2+}]_i$ response in the absence (replaced with 140 mM Li^+, grey) or presence of 140 mM extracellular Na^+ (black). B: Typical $[Ca^{2+}]_i$ changes after pretreatment with vehicle (black) or with 5 mM Ni^{2+} (grey) for 5 min.

induced Ca^{2+} increase was largely inhibited, but not completely, by pretreatment with 20 μM verapamil plus 5 μM thapsigargin.

Lack of involvement of Na^+–Ca^{2+} exchanger

Finally, we examined the involvement of the Na^+–Ca^{2+} exchanger in the LPC-induced Ca^{2+} response in SH-SY5Y cells in Ca^{2+}-containing medium. Replacing external Na^+ with Li^+ (140 mM) or 5-min pretreatment with 5 mM Ni^{2+}, a blocker of Na^+–Ca^{2+} exchanger, had no effect on LPC-induced Ca^{2+} response (Fig. 9).

Discussion

In the present study, we have characterized the pathway involved in $[Ca^{2+}]_i$ increase activated by LPC in human neuroblastoma SH-SY5Y cells. The results showed that LPC produced a sustained increase in $[Ca^{2+}]_i$, and this effect was induced by LPC itself but not its hydrolysis product, since GPC, a hydrolysis product of LPC by NTE-LysoPLA, could not cause any Ca^{2+} increase under the same experimental conditions. LPC-induced Ca^{2+} response was reduced by 36% in the absence of $Ca^{2+}{}_e$, and Ca^{2+} restoration experiments showed that there appeared to be an additional increase in $[Ca^{2+}]_i$ when Ca^{2+} was added back. In Ca^{2+}-free medium, LPC did not produce Ca^{2+} rise after depletion of ER Ca^{2+} stores by thapsigargin, indicating that LPC-induced Ca^{2+} rise in SH-SY5Y cells is dependent on intracellular Ca^{2+} stores. Together, these data suggest that Ca^{2+} from extracellular space and intracellular ER Ca^{2+} stores both contribute to LPC-induced Ca^{2+} increase, and that intracellular Ca^{2+} may play a more important role. In myocytes, the L-type channel may be responsible for the influx of extracellular Ca^{2+}, since pretreatment with verapamil, a specific blocker of L-type Ca^{2+} channel, blocked the increase in $[Ca^{2+}]_i$ caused by LPC (Sedlis et al., 1983). However, in rat cardiomyocytes, verapamil did not affect the LPC-induced $[Ca^{2+}]_i$ increase (Yu et al., 1998). In our studies, pretreatment of cells with verapamil attenuated the Ca^{2+} response by 23%, indicating that L-type Ca^{2+} channels are only partly responsible for Ca^{2+} influx in this cell line. We also investigated the Na^+–Ca^{2+} exchanger as another possible protein involved in the LPC-induced Ca^{2+} response (Yu et al., 1998; Karli et al., 1979). However, in our studies, pretreatment with Ni^{2+}, a blocker of Na^+–Ca^{2+} exchanger (O'Neill et al., 1988), or Na^+-deprivation did not affect the Ca^{2+} response to LPC, implying that Na^+–Ca^{2+} exchanger was not involved in the LPC response in SH-SY5Y cells.

Previously, it has been demonstrated that Ca^{2+} release from ER stores induced by LPC usually occurs through the $G_{i/o}/G_{q/11}$ G protein-phospholipase C-IP_3R pathway (Xu, 2002). However, in our studies, LPC-induced Ca^{2+} rise in Ca^{2+}-free medium is not blocked by 2-APB, an IP_3R inhibitor, suggesting that this effect of LPC is not mediated by activation of IP_3Rs in this cell line. Lack of any inhibitory effect of PTX, a known inhibitor of $G_{i/o}$ proteins, further confirmed this result, and thus the involvement of $G_{i/o}/G_{q/11}$ G protein-phospholipase C-IP_3R pathway was excluded. Besides IP_3Rs, RyRs are another class of intracellular Ca^{2+} releasing channels on ER. These receptors have also been shown to play an important role in eliciting increase in $[Ca^{2+}]_i$ induced by LPC in some studies (Yu et al., 1998; Vigne et al., 1990). Consistent with these results, pretreatment of cells with either 30 μM ruthenium red or 10 mM procaine, both specific inhibitors of the RyRs, completely blocked LPC-induced Ca^{2+} rise in Ca^{2+}-free medium. Therefore, we concluded that Ca^{2+} rise induced by LPC from Ca^{2+} stores may be through activation of RyRs on ER in SH-SY5Y cells.

The principal activator of RyRs is Ca^{2+} itself, and whether it can activate the channels is determined by intracellular Ca^{2+} releasing messengers, which are generated by stimuli binding to cell surface receptors on cell membrane (Berridge et al., 2000). LPC is a wedge-shaped molecule and it inserts readily into the outer monolayer of the cell membrane, but does not seem to flip quickly into the inner monolayer (Mohandas et al., 1982). Our experiments with digitonin-permeabilized cells showed that LPC could not induce any $[Ca^{2+}]_i$ rise in Ca^{2+}-free medium, in which RyRs are still functional since caffeine can induce Ca^{2+} rise in the permeabilized cells. This indicates that LPC cannot stimulate RyR directly and LPC-induced Ca^{2+} rise needs intact cell membrane. Thus we postulated that LPC might act on receptors on plasma membrane to generate mobilizing second messengers, which transfer the signal to activate RyRs on ER. Moreover, GPC, a hydrolysis product of LPC, is not the stimulatory molecular on RyRs since it could not cause any Ca^{2+} increase in digitonin-permeabilized cells (data not shown).

LPC causes a sustained increase in $[Ca^{2+}]_i$ and the increased Ca^{2+} level does not restore the resting level in the detective course. Since Na^+–Ca^{2+} exchanger was not involved in the LPC-induced Ca^{2+} response in this cell line, the phenomena indicated that LPC may inhibit Ca^{2+} extrusion of Ca^{2+} pumps on cell membrane or Ca^{2+} uptake in ER through SERCA, which is responsible for restoring the cytosolic Ca^{2+} to resting state. However, ATP hydrolytic activity of isolated ER preparation was not affected by 10 μM LPC (data not shown), which suggested that SERCA may be not involved in LPC-induced Ca^{2+} response. On the other hand, we found that combined blockade of L-type Ca^{2+} channels and inhibition of SERCA activity by verapamil and thapsigargin markedly inhibited but did not abolish the Ca^{2+} response to LPC, suggesting further complexity in the response elicited by LPC in SH-SY5Y cells. We can speculate that the sustained increase in $[Ca^{2+}]_i$ induced by LPC and this incomplete block of Ca^{2+} rise may result from inhibition of Ca^{2+} extrusion.

Reports suggest that LPC may cause demyelination, together with a variable degree of axonal degeneration, which is a common hallmark of several human diseases, including OPIDN (Kostrzewa and Segura-Aguilar, 2003; Iqbal et al., 2003; Johnson, 1969; Bouldin and Cavanagh, 1979). However, the exact biochemical mechanism(s) of LPC-induced demyelination and axonal degeneration remains unknown. Deregulation of neuronal Ca^{2+} homeostasis associated with the breakdown of the cytoskeletal elements is known to occur in different neurodegenerative disorders (Choudhary and Gill, 2001; Schlaepfer and Hasler, 1979; Schlaepfer, 1987). Calcium appears to contribute to such degenerative changes by facilitating the activities of Ca^{2+}-

associated kinases or proteases (Berlet, 1987; Suwita et al., 1986), and that perturbed ER-mediated Ca^{2+} regulation may play a central role in the neurodegenerative process (Yu et al., 1999; Mattson et al., 2000). In the present study, we found that Ca^{2+} from ER stores has an important role in LPC-induced Ca^{2+} overload. Therefore, we postulated that Ca^{2+} overload induced by LPC might be involved in the complex sequence of LPC-induced demyelination and axonal degeneration; moreover, this process may be linked to the excessive activation of ER. Elucidation of the mechanism of LPC-induced Ca^{2+} mobilization may contribute to understanding its effect of demyelination and axonal degeneration. In turn, this might shed light on the processes involved in OPIDN and some other human neurodegenerative diseases.

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