We studied the effects of the divalent cation ionophore A23187 on apoptotic signaling in MH1C1 cells. Addition of A23187 caused a fast rise of cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)), which returned close to the resting level within about 40 s. The [Ca\(^{2+}\)]\(_{i}\) rise was immediately followed by phospholipid hydrolysis, which could be inhibited by aristolochic acid or by pretreatment with thapsigargin in Ca\(^{2+}\)-free medium, indicating that the Ca\(^{2+}\)-dependent cytosolic phospholipase A\(_2\) (cPLA\(_2\)) was involved. These early events were followed by opening of the mitochondrial permeability transition pore (PTP) and by apoptosis in about 30% of the cell population. In keeping with a cause-effect relationship between addition of A23187, activation of cPLA\(_2\), PTP opening, and cell death, all events but the [Ca\(^{2+}\)]\(_{i}\) rise were prevented by aristolochic acid. The number of cells killed by A23187 was doubled by treatment with 0.5 \(\mu\)M MK886 and 5 \(\mu\)M indomethacin, which inhibit arachidonic acid metabolism through the 5-lipoxygenase and cyclooxygenase pathway, respectively. Consistent with the key role of free arachidonic acid, its levels increased within minutes of treatment with A23187; the increase being more pronounced in the presence of MK886 plus indomethacin. Cell death was preceded by cytochrome c release and cleavage of caspase 9 and 3, but not of caspase 8. All these events were prevented by aristolochic acid and by the PTP inhibitor cyclosporin A. Thus, A23187 triggers the apoptotic cascade through the release of arachidonic acid by cPLA\(_2\), in a process that is amplified when transformation of arachidonic acid into prostaglandins and leukotrienes is inhibited. These findings identify arachidonic acid as the causal link between A23187-dependent perturbation of Ca\(^{2+}\) homeostasis and the effector mechanisms of cell death.

Regulation of cell death by Ca\(^{2+}\) is a problem of great complexity. First proposed in the early 1970s as a mediator of cell demise in heart ischemia (1, 2), Ca\(^{2+}\) is today considered a key to both necrosis and apoptosis through its modulation of transcriptional factors, kinase and phosphatase signaling cascades, activation of enzymes involved in protein degradation and phospholipid metabolism, and modulation of mitochondrial function (3).

Mitochondria are central to Ca\(^{2+}\) signaling. This is true of both the response to physiological stimuli that pertains to normal cell function and the response to apoptotic signals that leads to cell demise (4). A key switch between cell survival and cell death is the mitochondrial PTP, a high conductance channel that may open in response to mitochondrial Ca\(^{2+}\) uptake (5). The PTP participates in the release of proapoptotic proteins through at least two mechanisms: swelling-dependent rupture of the outer mitochondrial membrane and remodeling of mitochondrial cristae, which increases the availability of cytochrome c for release through specific pathways activated by outer membrane tBid insertion (6, 7). PTP opening is not always followed by cell death, and the relationship between the two events appears to correlate with the pore open time. Only openings that last long enough to cause measurable depolarization are followed by cytochrome c release and detrimental effects on cell survival (8, 9).

A key aspect of PTP pathophysiology is that matrix Ca\(^{2+}\) is an essential permissive factor for pore opening, yet Ca\(^{2+}\) alone may not be sufficient. Indeed, only under conditions of overload, Ca\(^{2+}\) as such can trigger PTP opening in isolated mitochondria, an event that is also facilitated by the accompanying matrix alkalization (10). Under the conditions prevailing in situ, the PTP open-closed transitions are modulated by a large variety of additional factors that may critically affect the outcome of an identical Ca\(^{2+}\) signal (5). Many apoptotic signaling molecules act as PTP inducers, and these include lipid mediators like ceramides (11, 12), GD3 ganglioside (13–16), and arachidonic acid (17–19). The latter is particularly interesting in the context of Ca\(^{2+}\)-dependent cell death. Indeed, it has been shown that arachidonic acid-selective, Ca\(^{2+}\)-dependent cPLA\(_2\) is essential for the cytotoxic action of tumor necrosis factor \(\alpha\) (20), which triggers PTP opening (21) through activation of phospholipid hydrolysis (17) and that arachidonic acid signals apoptosis (22, 23) through a mitochondrial effect that can be amplified by inhibition of LOX and COX (18).

The abbreviations used are: PTP, permeability transition pore; bis-BODIPY®-FL-CL\(_{3}\), 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diazacyclopenta-1,3-cyclooctadiene-3-undecanoyl)-3-glycero-3-phosphocholine; [Ca\(^{2+}\)]\(_{i}\), cytosolic Ca\(^{2+}\) concentration; COX, cyclooxygenase; cPLA\(_2\), cytosolic phospholipase A\(_2\); CaS, cyclosporin A; HBSS, Hank’s balanced salt solution; LOX, lipoxygenase; MH1C1, Morris Hepatoma of the rat clone 1C1; MK886, 3-[(tert-butylsulfonyl)iminomethyl]-1-(4-chlorobenzoyl)-5-isopropyl-IH-indol-2-yl]-2,2-dimethylpropionic acid; HETE, hydroxyeicosatetraenoic acid; AM, acetomethoxy ester.
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Addition of A23187 should allow Ca²⁺ equilibration with an increase of [Ca²⁺]ₘ, which should mimic the Ca²⁺ overload taking place under pathological conditions at proper concentrations and incubation times (24–31). Unexpectedly, however, we found that addition of A23187 to MH1C1 cells caused a slow mitochondrial depolarization and death of about 30% of cells, findings that are inconsistent with the expected consequences of cellular Ca²⁺ overload (9). Here we show that addition of A23187 caused as fast but only transient rise of [Ca²⁺]ₘ, which was immediately followed by a rapid increase of cPLA₂ activity and the PT-P opening. The latter event occurred during the time when [Ca²⁺]ₘ had already returned to nearly basal levels. We identified the proapoptotic signal triggered by A23187 as free arachidonic acid, whose levels could be dramatically increased by treatment with the LOX inhibitor MK886 plus the COX inhibitor indomethacin. A23187-triggered cell death was preceded by PTP opening, cytochrome c release, and cleavage of caspase 9 and 3, but not of caspase 8. All these events except for the transient rise of [Ca²⁺]ₘ, were blocked by the cPLA₂ inhibitor arachidonic acid or by the PTP inhibitor CsA. Thus, the major effect of the [Ca²⁺]ₘ increase caused by A23187 is activation of cPLA₂ with generation of arachidonic acid, which causes apoptosis exclusively through the mitochondrial pathway. This signaling pathway is amplified when transformation of arachidonic acid into prostaglandins and leukotrienes has been inhibited.

EXPERIMENTAL PROCEDURES

Cell Cultures—MH1C1 cells were grown in Ham’s F-10 nutrient mixture supplemented with 10% fetal calf serum in a humidified atmosphere of 95% air, 5% CO₂ at 37°C in a Forma tissue culture water-jacketed incubator. For experiments of fluorescence microscopy, cells were seeded onto uncoated 24-mm (for calcein, fluo-3, bis-BODIPY-FL-C₁₁-PC, and annexin V staining) or 13-mm (for immunofluorescence) diameter round glass coverslips (50,000 cells/coverslip) and grown for 2 days as described above.

Fluo-3 Staining and Imaging—Cells were loaded with 5 μM fluo-3 AM (acetoxymethoxy ester) for 1 h at 37°C in 1 ml of bicarbonate- and phenol red-free HBSS supplemented with 10 mM Hepes, pH 7.4, 0.005% pluronic acid, and 2 μM cyclosporin H (CsH) (32). Cells were then washed free of fluo-3 and pluronic acid and maintained in HBSS-Hepes containing 2 μM CsH. Cellular fluorescence images were acquired with an Olympus IMT-2 inverted microscope, equipped with a xenon light source (Hamamatsu G-17), and fluorescence microscopy was carried out and analyzed using Metamorph software. Sequential digital images were acquired and analyzed using Metamorph software. Sequential digital images were acquired at the times indicated in Fig. 2, and the total fluorescence of 10 cells in each field was recorded and stored. The data were corrected for background fluorescence, and initial fluorescence values are normalized for comparative purposes.

Gas Chromatography and Mass Spectrum Analysis—Cell extracts were taken to complete dryness with a gentle stream of N₂ gas and then 10 μl of CHCl₃ were added to give a homogeneous solution, and 1 μl was injected in the gas chromatograph. Analysis was carried out and analyzed using Metamorph software. Sequential digital images were acquired at the times indicated in Fig. 2, and the total fluorescence of 10 cells in each field was recorded and stored. The data were corrected for background fluorescence, and initial fluorescence values are normalized for comparative purposes.

Lipid Analysis—Cells were treated with A23187 and other reagents as described in the legend to Figs. 8 and 9, and they were extracted with chloroform/methanol exactly as described in Ref. 33 except that homogenization was carried out on ice for 15 min, and the sample was not filtered. The lipid extracts were subjected to thin layer chromatography on 10 × 10 cm plates (Merck, Darmstadt n. 5641) as specified in the figure legends, and the migration of specific lipids was identified with authentic standards (Avanti polar lipids). The plates were sprayed with 20% (v/v) H₂SO₄, and the spots were visualized by heating.

Cytochrome c Release and Cell Death—For staining with annexin V, each coverslip was incubated for 15 min at 25°C in 0.5 ml of a solution containing 140 mM NaCl, 5 mM CaCl₂, and 10 mM Hepes-NaOH, pH 7.4, 2 μM propidium iodide (Sigma) and annexin V-FLUOS (Roche Applied Science) to a final dilution of 1:25 (v/v). Cells were washed twice with HBSS-Hepes before analysis. Cellular fluorescence images were acquired with the Olympus IMT-2 inverted microscope exactly as described (8). Immunodetection of the relative distribution of the bc complex and of cytochrome c was carried out and analyzed as described in Ref. 8. The analysis yields a “localization index” of 1 when the distribution of cytochrome c matches that of the bc complex, whereas the index decreases as the distribution of cytochrome c becomes more diffuse (9). Caspase Cleavage—MH1C1 cells were grown to near confluence in 25-cm² tissue culture flasks. The flask were rinsed with serum-free medium, cells were withdrawn, pooled with the medium and the cell density was measured. Variable proportion of cells, at the end of the experiment the medium with floating cells was removed from each flask and transferred to a 25-cm² tissue culture flask containing 2 ml polyethylene glycol to be used as substrate for adherent cells from the same flask. The cells were then rinsed with phosphate-buffered saline and treated with 0.5 ml of trypsin (0.05%), w/v) plus EDTA (0.02%, w/v) for 2 min. Following the addition of 4.5 ml of serum-free medium, cells were withdrawn, pooled with the medium previously removed from each flask, and collected by centrifugation at 1,000 × g for 5 min. The supernatant was removed and the cell pellet was dissolved in 1 ml of ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7,5, 1% Nonidet P-40, 1 μg ml⁻¹ phenylmethylsulfonyl fluoride, 1 μg ml⁻¹ pepstatin, and 1 μg ml⁻¹ leupeptin). After 30 min at 4°C, the extracts were cleared by centrifugation at 14,000 × g at 4°C.
for 10 min. The protein concentration of each extract was determined with the Bradford reagents (Bio-Rad), and equal protein amounts were precipitated with 4 volumes of ice-cold acetone for 10 min followed by centrifugation at 14,000 \( \times g \). The acetone was removed, the samples were air-dried at room temperature, and the pellets were finally dissolved by boiling for 5 min in Laemmli gel sample buffer containing 5% 2-mercaptoethanol. Proteins were resolved by SDS-PAGE on 1.5-mm thick 12% acrylamide-0.4% bisacrylamide minigels, transferred to nitrocellulose, and probed with antibodies against caspases 8 and 9 (Santa Cruz Biotechnology), and 3 (Cell Signaling; they all recognized caspase 9 plus 3 cleavage, respectively. The [Ca\(^{2+}\)]\(_i\) transient induced by A23187 was reduced in height but not abolished by pretreatment with 2 \( \mu \)M thapsigargin for 30 min or by incubation in Ca\(^{2+}\)-free medium containing 0.1 mM EGTA (results not shown). However, it was completely blunted by a combination of the two treatments (Fig. 1, trace b). These findings indicate that both extracellular Ca\(^{2+}\) and intracellular Ca\(^{2+}\) stores contribute to the [Ca\(^{2+}\)]\(_i\) transient induced by A23187. The [Ca\(^{2+}\)]\(_i\) response depicted in Fig. 1 was observed in all treated cells, yet A23187 caused cell death only in about 30% of the population (9) (see also Fig. 4 below). Taken together, these findings suggest that the initial [Ca\(^{2+}\)]\(_i\) rise may have generated additional signal(s) causing cell death in a subpopulation of sensitive cells.

**Effects of A23187 on Phospholipid Hydrolysis and PTP Opening**—We next investigated whether A23187 could initiate early phospholipid hydrolysis. To this end, cells were enriched with the synthetic phospholipid bis-BODIPY-FL-C11-PC, which undergoes dequenching (with a corresponding fluorescence increase) upon cleavage of the labeled phospholipid in position 2. It can be seen that the addition of 2 \( \mu \)M A23187 caused a mostly intracellular fluorescence increase (Fig. 2, panel B), in keeping with the recent demonstration that cPLA\(_2\) translocates to intracellular membranes after its activation (34–37). Consistent with activation of the Ca\(^{2+}\)-dependent cPLA\(_2\), phospholipid hydrolysis triggered by A23187 was inhibited by aristolochic acid (38–40) or by treatment with thapsigargin in the presence of EGTA, but not by the PTP inhibitor CsA (Fig. 2, panel C). Note that the process of phospholipid hydrolysis detected by bis-BODIPY-FL-C11-PC immediately followed the [Ca\(^{2+}\)]\(_i\) rise induced by A23187 (Fig. 2, panel C, compare with the inset in Fig. 1). It must be stressed that neither aristolochic acid nor CsA modified the extent or kinetics of the [Ca\(^{2+}\)]\(_i\) response to A23187 (results not shown).

We have previously shown that treatment of MH1C1 cells with tumor necrosis factor \( \alpha \) activates phospholipid hydrolysis followed by PTP opening (17). We tested whether PLA\(_2\) activation by A23187 caused PTP opening with the sensitive calcine loading Ca\(^{2+}\)-quenching technique (8). The experiments shown in Fig. 3, panel A show that treatment with 2 \( \mu \)M A23187 was
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A23187 Induces Apoptosis through the Mitochondrial Pathway, Which Is Amplified by MK886 Plus Indomethacin—Despite the demonstrable increase of the PTP open time, Fig. 4 shows that A23187 caused the death of less than 30% of a population of MH1C1 cells within 5 h, see also Ref. 9. Cell killing was largely inhibited by aristolochic acid and CsA, indicating that phospholipid hydrolysis and PTP opening were involved in activation of the death program in a subpopulation of sensitive cells (Fig. 4). Because the cytotoxic effects of added arachidonic acid can be amplified by treatment with MK886 (a 5-LOX inhibitor) plus indomethacin (a general COX inhibitor) (18), we tested whether the fraction of cells responding to apoptosis to treatment with A23187 could be increased by MK886 and/or indomethacin. At the concentrations used in this study, neither MK886 nor indomethacin alone caused direct, short term toxicity to MH1C1 cells, nor did they increase the fraction of cells entering the apoptotic program upon stimula-
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...tion with A23187 (Fig. 4). When used in combination, however, MK886 and indomethacin increased the fraction of cells killed by A23187 to over 60%, in a process that maintained its sensitivity to both CsA and aristolochic acid (Fig. 4). It must be mentioned that neither MK886 nor indomethacin affected the [Ca²⁺]i, and bis-BODIPY-FL-C11-PC response to A23187 (results not shown).

To assess the pathway(s) for cell death we studied the kinetics of cytochrome c release and the pattern of caspase 8, 9, and 3 cleavage under the various conditions of the present study. A23187 induced release of cytochrome c that was potentiated by treatment with MK886 and indomethacin (Fig. 5, panel A) in a process that maintained its sensitivity to both aristolochic acid and CsA (Fig. 5, panel B). Consistent with selective activation of the mitochondrial pathway via cPLA₂ and the PTP: (i) A23187 caused cleavage of caspase 9 (Fig. 6, middle panel) and caspase 3 (Fig. 6, lower panel), which could be detected after 16 but not after 5 h of incubation (Fig. 6, compare lane 4 with lane 1), but not of caspase 8 (Fig. 6, upper panel); (ii) in A23187-treated cells, MK886 plus indomethacin-potentiated cleavage of caspase 9 (Fig. 7, middle panel) and caspase 3 (Fig. 6, lower panel), which could be detected after 5 h of incubation (Fig. 7, compare lane 10 with lanes 4 and 7), whereas cleavage of caspase 8 was not observed (Fig. 7, upper panel); (iii) both aristolochic acid (Figs. 6 and 7, lanes labeled A) and CsA (Figs. 6 and 7, lanes labeled C) fully prevented cleavage of caspases 9 and 3.

A23187 Causes Accumulation of Free Arachidonic Acid in a Process Enhanced by MK886 Plus Indomethacin and Inhibited by Aristolochic Acid—Based on the pharmacological experiments reported so far (i.e. inhibition of cell death by aristolochic acid and its stimulation by MK886 and indomethacin) we hypothesized that the mechanism underlying A23187 toxicity was an increase of free arachidonic acid. To test this hypothesis, we studied the cellular lipid composition before and after treatment with A23187 by chloroform/methanol extraction followed by thin layer chromatography with chloroform (60%), methanol (30%), formic acid (7%), and H₂O (3%). The experiments of Fig. 8, panel A document that treatment with A23187 caused phospholipid hydrolysis, with appearance of lysophosphatidylcholine and lysophosphatidylethanolamine (the signal of lysophosphatidylserine could not be resolved from that of sphingomyelin). The lysophospholipids were detectable within 5 min of the addition of A23187 (panel A, lane 2), and their amount increased over the 30-min time frame of the experiment (panel A, lanes 2–4). Consistent with the experiments where phospholipid hydrolysis was followed by bis-BODIPY-FL-C11-PC (Fig. 2), formation of lysophospholipids was completely prevented by pretreatment with aristolochic acid (panel A, lane 6). An identical pattern of lysophospholipid formation was detected in cells pretreated with MK886 plus indomethacin (panel A, lanes 7–11). Analysis of the same samples with chloroform 95%, formic acid 5% as the eluent allowed resolution of the fatty acids, which accumulated with the same time course as the lysophospholipids after the addition of A23187 (panel B, lanes 2 and 3). Consistent with a key role of LOX and COX in fatty acid metabolism, the addition of MK886 and indomethacin greatly increased the amount of fatty acids detectable after treatment with A23187 (panel B, lanes 7 and 8). Finally, fatty acid production was completely blocked by aristolochic acid (panel B, lanes 5 and 9). The nature of the fatty acid(s) produced by phospholipid hydrolysis was determined by gas chromatography and mass spectroscopy of the lipid extracts. Only one species was detected after treatment...
with A23187 (Fig. 9, panel A), which had the same retention time as authentic arachidonic acid (not shown). The mass spectrum unequivocally identified the typical fragmentation pattern of arachidonic acid (304 m/z, 2%; 79, 100%; 91, 98%; 119, 55%; 147, 35%) (Fig. 9, panel B), and no other species could be detected throughout elution of the peak. A time course analysis revealed that upon addition of A23187 the peak concentration of arachidonic acid was reached at 15 min, with a decline at 30 min (Fig. 9, panel C). In the presence of MK886 plus indomethacin the concentration of arachidonic acid was about 10-fold higher, and continued to increase throughout the 30-min time course of the experiment (Fig. 9, panel C). Free arachidonic acid could still be detected in the extracts after 5 h of incubation with A23187 both in the absence and presence of MK886 plus indomethacin (results not shown).

DISCUSSION

The divalent cation ionophore A23187 was introduced in biomedical research in the early 1970s (41). It proved very useful to study mitochondrial ion transport (42, 43) and intracellular Ca²⁺ homeostasis (44), and as a tool to investigate Ca²⁺-dependent processes like activation of cPLA₂ (45), arachidonic acid metabolism (46), and induction of cell death (24–31). Our work is generally consistent with previous reports on the cellular and mitochondrial effects of A23187 in situ (25–31), but it provides unique clues on the role of arachidonic acid released by cPLA₂ activation as the mechanistic link between A23187-dependent perturbation of Ca²⁺ homeostasis and the effector mechanisms of cell death.

The major findings of the present article are summarized in Fig. 10. We have established that a Ca²⁺-dependent PLA₂ plays an essential role in the proapoptotic effects of A23187 in MH1C1 cells. Indeed, the rise of [Ca²⁺]ᵢ following the addition of A23187 was transient; and it did not cause mitochondrial PTP opening and apoptosis as a direct consequence of Ca²⁺ overload. Rather, PTP opening, release of cytochrome c, activation of caspases 9 and 3, and cell death in a subset of sensitive cells were all initiated by hydrolysis of membrane phospholipids. It appears that the key enzyme involved in triggering these events is a phospholipase, the most likely candidate being cPLA₂ based on the following: (i) the inhibitory effects of aristolochic acid (38–40) and Ca²⁺ dependence (34) and (ii) the lack of expression of soluble PLA₂ in our cells (results not shown). Direct measurements have demonstrated that treatment with A23187 causes a selective increase of free arachidonic acid, which is further enhanced by the addition of MK886 (to inhibit 5-LOX) and indomethacin (to inhibit COX-1 and -2). The proapoptotic effects of A23187 are caused by the increase of free arachidonic rather than by the increase of [Ca²⁺]ᵢ, as such. Indeed, and irrespective of the presence of MK886 plus indomethacin, all the effects of A23187 (but Ca²⁺ rise) were blocked by aristolochic acid, and all the effects of A23187 (but Ca²⁺ rise and cPLA₂ activation) were blocked by the PTP inhibitor CsA. These findings fully support the scheme presented in Fig. 10, which highlights the key role of mitochon-
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...protein phosphatase activity that is downstream of cPLA$_2$ activation...
Arachidonic Acid Released by Phospholipase A2 Activation Triggers Ca^{2+}-dependent Apoptosis through the Mitochondrial Pathway
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