Inhibition of Permeability-dependent Ca\(^{2+}\) Release from Mitochondria by N-Acylethanolamines, a Class of Lipids Synthesized in Ischemic Heart Tissue*

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Long chain N-acylethanolamines which accumulate in large amounts in the ischemic areas of canine myocardium (Epps, D. E., Schmid, P. C., Natarajan, V., and Schmid, H. H. O. (1979) *Biochem. Biophys. Res. Commun.* 90, 628-633) are shown here to inhibit the development of increased inner membrane permeability in heart mitochondria produced by Ca\(^{2+}\) plus Ca\(^{2+}\)-releasing agents such as oxalacetate, N-ethylmaleimide, and palmitoyl coenzyme A. The inhibition is concentration-dependent, requiring approximately 30 \(\mu\)M for a half-maximal effect with N-oleoyl ethanolamine. Higher levels of this compound inhibit energy-dependent Ca\(^{2+}\) accumulation, maximal rates of succinate oxidation and the development of membrane potential. Half-maximal effects for these activities are seen at approximately 120 \(\mu\)M. Inhibition of Ca\(^{2+}\) uptake appears to be a secondary consequence of inhibited energy production rather than an effect on the Ca\(^{2+}\) uniport per se. Inhibition of succinate oxidation is noncompetitive with respect to succinate concentration, suggesting that this activity arises at the level of electron transport.

N-Acylethanolamine has analogous actions on liver mitochondria except that 2- to 3-fold lower concentrations are required to inhibit energy production. In addition, at lower levels, a stimulation rather than inhibition of Ca\(^{2+}\)-dependent permeability increase is observed. This difference is due to the action of a hydrolyase degrading the amide linkage, an enzyme which is present in liver but not in heart mitochondria. The resulting accumulation of free fatty acids in liver mitochondria can lead to the synthesis of intramitochondrial acylcoenzyme A which increases the sensitivity to Ca\(^{2+}\) and other Ca\(^{2+}\)-releasing agents. A survey of tissue homogenates revealed that hydrolysis of N-oleoyl ethanolamine occurs most rapidly in liver but does not occur in heart.

Considering the actions of N-acylethanolamine on mitochondria and the requirements for the biosynthesis of these compounds, it is concluded that they could function to protect cells subjected to ischemic insult and perhaps to injury by other agents which provoke large increases in intracellular Ca\(^{2+}\) levels.

Mitochondria from liver and heart release accumulated Ca\(^{2+}\) when incubated in the presence of a variety of agents and compounds (Ca\(^{2+}\)-releasing agents) which can be broadly divided into two classes. The first class, which includes such agents as ruthenium red (1, 2), EGTA\(^3\) (3, 4), Na\(^+\) (5, 6), and H\(^+\) (7, 8), causes Ca\(^{2+}\) release seemingly without altering the permeability of the inner membrane or collapsing the mitochondrial membrane potential. The second class, including such compounds as N-ethylmaleimide, oxalacetate, palmitoyl coenzyme A, and inorganic phosphate, causes Ca\(^{2+}\) release by promoting, together with intramitochondrial Ca\(^{2+}\), a non-specific increase in the permeability of the inner mitochondrial membrane (9-13).

We have postulated that the permeability change is produced through an activation of intramitochondrial phospholipase A\(_2\) with a concomitant inhibition of acyl-coenzyme A-lysophospholipid acyltransferase activity. This would lead to an intramembrane accumulation of lysophospholipids and free fatty acids which, in turn, are proposed to produce the increased permeability (9-12).

Under *in vitro* conditions, the permeability-dependent mechanism of Ca\(^{2+}\) release can lead to the complete inhibition of energy-linked functions and disruption of mitochondrial structure which poses questions as to the physiological relevance of the process. However, mitochondria which have become permeable due to the action of Ca\(^{2+}\) and a Ca\(^{2+}\)-releasing agent, have functional and ultrastructural properties which resemble those of mitochondria obtained from pathological tissues, notably from tissues subjected to ischemic insult (14-18). Thus, the phospholipase A\(_2\)-dependent increase in inner membrane permeability may be a reflection of the mechanism by which mitochondria are damaged in ischemic tissue.

We recently reported that N-acyl ethanalamines accumulate in ischemic areas of canine myocardium to levels as high as 500 nmol/g of tissue (19, 20). The biosynthesis of these compounds involves an initial N-acylation of phosphatidyethanolamine or lysophosphatidylethanolamine, a reaction which requires Ca\(^{2+}\) in the millimolar concentration range, followed by degradation of the product to the N-acyl ethanolamine (21, 22). The amide-linked fatty acids consist almost exclusively of palmitic, stearic, oleic, and linoleic acids (19, 20). N-Acylethanolamines have known pharmacological activities, particularly anti-inflammatory properties (23), which led to the hypothesis...
that accumulation of these agents in infarcted heart tissue may represent a component of cellular defense mechanisms against the degradative changes brought about by ischemia. In the present communication, we demonstrate that these compounds inhibit Ca$^{2+}$ release from mitochondria induced by the second class of Ca$^{2+}$-releasing agents, by preventing the increase in inner membrane permeability which is responsible for their actions. It is also shown that N-acyl ethanolamines inhibit electron transport from succinate, limit the development of membrane potential, and inhibit the accumulation of Ca$^{2+}$ by mitochondria. These properties suggest that the compounds play important roles in the metabolism of injured cells.

**Materials and Methods**

Preparation and Incubation of Mitochondria—Heart and liver mitochondria were prepared from male Sprague-Dawley rats weighing approximately 250 g, which had been provided with rat chow and water ad libitum prior to killing. Subsarcolemmal and interfibrillar heart mitochondria were separated as described by Palmer et al. (24) with the exception that the 3 mM 4-morpholinopropanesulfonic acid buffer was replaced by 3 mM Hepes throughout the procedure. Liver mitochondria were prepared as previously described (11). Protein concentration was determined by the Biuret method after solubilization of the mitochondria with deoxycholate (25). Bovine serum albumin was utilized as the protein standard.

The incubation conditions are specified in the figure and table legends. N-Acylethanolamines were added to mitochondrial suspensions from stock solutions in ethanol. Uptake and release of Ca$^{2+}$ and changes in mitochondrial membrane potential were monitored by use of the indicating dyes antipyrylazo III (26) and safranin O (27), respectively. Changes in the mitochondrial NAD(P)$^+$NAD(P)H ratio were monitored by difference absorbance measurements at 340 versus 450 nm. Dual wavelength absorbance measurements were carried out with an Aminco DW2a spectrophotometer. Release of endogenous mitochondrial Mg$^{2+}$ was monitored by atomic absorption analysis of supernatants following sedimentation of the mitochondria at 15,000 $\times g$ in a microcentrifuge. Mitochondrial oxygen consumption, H$^+$ movements, and swelling were monitored as described previously (11).

Synthesis and Hydrolysis of N-Acylethanolamines—N-Acylethanolamines containing fatty acids of desired chain length and double bond position(s) and configurations were synthesized as described by Roe et al. (28), with modifications. The reactions were terminated by a 20% sodium bicarbonate extraction (29) in which the upper phase contained 10% (w/v) sodium bicarbonate to reduce the organic phase concentration of unreacted free fatty acid. The lower phase was concentrated with a rotary evaporator and chromatographically pure (Silica Gel H, hexane:ethyl acetate:acetic acid, 30:50:20, by volume). This material was recrystallized twice from ethanol, after which it was chromatographically pure (Silica Gel H, hexane:ethyl ether:acetone:acetic acid, 30:50:20:1, by volume). The identity of the products was confirmed by gas chromatography-mass spectrometry and infrared spectral analysis (20). N-[U-14C]Oleoyl-CoA, a product of interest, was prepared according to the procedure of Bachur and Udenfriend (30) to yield a final specific activity of 0.282 mCi/m mol.

Hydrolysis of the amide linkage in N-[U-14C]Oleoyl-CoA by mitochondrial or by tissue homogenates was investigated by monitoring the release of $^{14}$C-labeled oleic acid. For mitochondria, incubations were conducted in the media described in the legends to Figs. 3 and 4 for organelles from heart and liver, respectively. In addition, the media contained 80 $\mu$M N-[U-14C]Oleoyl-CoA. Reaction was initiated by the addition of mitochondria at 1.0 mg of protein/ml. At specified times, hydrolysis was terminated by extraction (29). Following the addition of unlabeled oleic acid as carrier, [$^{14}$C]oleic acid was separated from the labeled substrate by thin layer chromatography (Silica Gel H, chloroform:methanol:NH$_4$OH, 80:20:2 by volume). The area containing labeled and carrier oleic acid was visualized in iodine vapor, collected into vials, and the product quantitated by scintillation counting in a mixture composed of 1 ml of water and 10 ml of a toluene:Triton X-100 (2:1) mixture containing Permabead (Packard) at 4 g/liter.

For tissue homogenates, the determinations of N-[U-14C]Oleoyl-CoA oleoyl ethanolamine hydrolys were carried out in an equivalent manner except that succinate and rotenone were deleted from the media. Tissues were homogenized thoroughly with 10 volumes of 230 mM mannitol, 70 mM sucrose, 10 mM Hepes (Na$^+$), pH 7.4, utilizing a Polytron homogenizer (Brinkman). Aliquots of the homogenates sufficient to give a final protein concentration of 1.0 mg/ml were added to the homogenizing media containing the radiolabeled substrate, and hydrolysis was monitored as described above for mitochondria.

**Results**

Inhibition of Ca$^{2+}$ Release by N-Acylethanolamines—Fig. 1 demonstrates that release of accumulated Ca$^{2+}$ from subarcoclemmal heart mitochondria induced by oxalacetate is largely prevented by the presence of 80 $\mu$M N-oleoylthanolamine. As shown previously (11, 12), Ca$^{2+}$ release induced by oxalacetate results from an increased permeability of the inner membrane which also produces a release of the endogenous Mg$^{2+}$, collapse of the membrane potential, and other permeability-dependent phenomena. The numbers associated with individual traces in Fig. 1 show the extent of Mg$^{2+}$ release (in nanomoles per mg of protein) at the times indicated. The fact that the presence of N-oleoylthanolamine inhibits Ca$^{2+}$ and Mg$^{2+}$ release to a comparable extent (Fig. 1) and delays the collapse of membrane potential for a similar length of time (data not shown), indicates that it acts by preventing the increase in inner membrane permeability.

Previous work has shown that both Ca$^{2+}$ and oxalacetate must initially accumulate in the matrix space to effect increased inner membrane permeability and release of the accumulated ion (12). While it is clear from the data in Fig. 1 that N-oleoylthanolamine reduces the rate of Ca$^{2+}$ uptake, it does not prevent accumulation under these conditions, thus ruling out this possible explanation for its activity in preventing increased permeability. It remains possible, however, that the inhibitory agent acts by limiting the accumulation of the.
releasing agent. Fig. 2 demonstrates that this is not the case. When the intramitochondrial concentration of oxalacetate is increased, the ratio of reduced to oxidized pyridine nucleotides is decreased due to equilibration of this ratio with the malate/oxalacetate ratio through the action of malate dehydrogenase. Under conditions where the matrix pH is not altered, the oxidation of NAD(P)H upon addition of oxalacetate reflects the extent to which the matrix concentration of the compound is increased (12). Since upon addition of oxalacetate in the absence of Ca\textsuperscript{2+}, the extent of NAD(P)H oxidation is the same in the presence or absence of N-oleoylethanolamine (Fig. 2A), it does not appear that the inhibitory action of this compound on Ca\textsuperscript{2+} release arises from diminishing the matrix accumulation of the exogenous releasing agent.

This indication is strengthened by the data shown in Fig. 2B. The solid trace shows the extent of pyridine nucleotide oxidation which occurs when the inner membrane is made fully permeable by the addition of oxalacetate to Ca\textsuperscript{2+}-loaded mitochondria. The extent of oxidation is greater than in panel A since the concentration of oxalacetate is fully equilibrated across the membrane and the pH gradient has collapsed (11, 12). When the mitochondria are loaded with Sr\textsuperscript{2+} rather than Ca\textsuperscript{2+}, subsequent addition of oxalacetate fails to produce a permeable inner membrane since this cation is relatively ineffective in activating the phospholipase A\textsubscript{2} (9, 12). The dotted line in Fig. 2B shows that under these conditions the extent of pyridine nucleotide oxidation is the same as in Fig. 2A where no exogenous divalent cation was present. In this case, the presence of N-oleoylethanolamine clearly retards the rate of pyridine nucleotide oxidation (dashed line), but again, it does not alter the final extent of oxidation, indicating that the change in matrix oxalacetate concentration is unaffected by its presence.

Further indications that N-oleoylethanolamine inhibits oxalacetate-induced Ca\textsuperscript{2+} release by preventing the underlying, causative increase in inner membrane permeability were obtained from experiments of the type shown in Fig. 1 where it was found that it also inhibits Ca\textsuperscript{2+} and Mg\textsuperscript{2+} release induced by N-ethylmaleimide and palmitoyl coenzyme A (data not shown). These agents, like oxalacetate, induce Ca\textsuperscript{2+} release by promoting a phospholipase A\textsubscript{2}-dependent permeability increase (11, 12).

The extent of inhibition of Ca\textsuperscript{2+} release induced by oxala-
Inhibition of Ca\(^{2+}\) Release by N-Acylethanolamines

Effects of N-Acylethanolamines on Swelling and Ca\(^{2+}\) Transport in Liver Mitochondria—Since Ca\(^{2+}\)-releasing agents such as oxalacetate appear to act on heart and liver mitochondria in an equivalent manner (11, 12), we anticipated that N-acylethanolamines would also inhibit the development of increased permeability and release of Ca\(^{2+}\) from liver mitochondria. However, the action of these compounds on the liver organelles was found to be more complex. Fig. 4 shows the effects of increasing concentrations of N-oleoylethanolamine on the large amplitude swelling of liver mitochondria associated with permeability-dependent Ca\(^{2+}\) release. The data demonstrate that low concentrations of the compound result in a marked stimulation of the swelling rate produced by Ca\(^{2+}\) plus phosphate or Ca\(^{2+}\) plus oxalacetate while higher concentrations result in equivalent and complete inhibition of swelling induced by both agents.

It was further found as shown in Fig. 5A that the N-oleoylethanolamine by itself has complex effects on the uptake and retention of Ca\(^{2+}\) by the liver organelles. Under the incubation conditions employed, these mitochondria accumulate Ca\(^{2+}\) and retain it for an extended period with the onset of release corresponding with the attainment of anaerobiosis (Fig. 5A, absence of N-oleoylethanolamine). Low levels of N-oleoylethanolamine have little effect on the initial uptake of Ca\(^{2+}\) but dramatically shorten the time for which the accumulated cation is retained. As the concentration of the inhibitor is increased between 20 and 80 \(\mu\)M, the range during which the swelling rates in Fig. 4 become inhibited to approximately 10% of the maximal rate, the effect of N-oleoylethanolamine on Ca\(^{2+}\) uptake and retention is altered qualitatively. The compound does not markedly inhibit the extent of initial Ca\(^{2+}\) uptake, but shortens the retention time in a manner which is progressive as the inhibitor concentration is increased. The onset of Ca\(^{2+}\) release is not as abrupt as it is at lower concentrations or in the absence of the N-acylamide, but occurs at a progressively accelerating rate, characteristic of increased permeability and release of Ca\(^{2+}\) from liver mitochondria.

Fig. 5. The effect of N-oleoylethanolamine on Ca\(^{2+}\) uptake and retention by mitochondria. Liver and subsarcolemmal heart mitochondria were incubated as described in the legends to Figs. 4 and 3, respectively. The traces presented are difference absorbance measurements of antipyrilazo III which was present at 100 \(\mu\)M. CaCl\(_2\) was added at 100 nmol/mg of protein after a 2-min preincubation. The numbers associated with the individual traces indicate the concentrations of N-oleoylethanolamine which were present in the medium from the beginning of the experiment. A, liver mitochondria. At 0, 10, and 20 \(\mu\)M N-oleoylethanolamine, the onset of Ca\(^{2+}\) release occurred at 21, 12, and 10 min, respectively. B, heart mitochondria. In the absence of N-oleoylethanolamine, the onset of Ca\(^{2+}\) release occurred at 12 min.
of an autocatalyzed process. As the concentration of the inhibitor is increased further, the rate and extent of initial Ca\(^{2+}\) uptake declines rapidly; however, that portion which is accumulated is more readily retained than it is at the intermediary concentrations. The effects of N-oleoylethanolamine alone on Ca\(^{2+}\) uptake and retention by heart mitochondria (Fig. 5B) show several important differences from what is observed with liver mitochondria. Little effect of the compound is apparent in the 0 to 100 \(\mu\)M concentration range. Higher concentrations again inhibit Ca\(^{2+}\) uptake, although 3- to 4-fold higher levels are required. Ca\(^{2+}\) is retained until anaerobiosis (determined from parallel experiments) throughout the concentration range tested. It is thus apparent that intermediary levels of the N-acylamide do not provoke the progressive autocatalyzed Ca\(^{2+}\) release that is seen with liver mitochondria.

The Effect of N-Oleoylethanolamine on Permeability, Membrane Potential, and Maximal Rates of Electron Transport—Insight into the basis of the complexity of N-acyl ethanolamine effects on permeability changes and Ca\(^{2+}\) transport in liver mitochondria and the differences in these effects in heart mitochondria is obtained by examining its effects on Mg\(^{2+}\) retention and on parameters which reflect the capacity of the organelles to carry out energy-dependent functions. Fig. 6 demonstrates that in the concentration range where N-oleoyl ethanolamine alone affects the uptake and retention of Ca\(^{2+}\) by liver mitochondria, it also affects the retention of endogenous Mg\(^{2+}\). Between 0 and approximately 40 \(\mu\)M, N-oleoyl ethanolamine results in loss of a substantial portion of endogenous Mg\(^{2+}\) from Ca\(^{2+}\)-loaded mitochondria, indicating that it promotes increased inner membrane permeability. However, as the concentration of the compound is increased, the extent of Mg\(^{2+}\) release is diminished similar to what was seen in Fig. 4 for the effects of the agent on Ca\(^{2+}\) plus phosphate- or Ca\(^{2+}\) plus oxalacetate-induced swelling. The compound does not induce Mg\(^{2+}\) release from liver mitochondria which were not loaded with endogenous Ca\(^{2+}\), indicating that Mg\(^{2+}\) loss does not reflect a simple detergent action of N-oleoyl ethanolamine. Fig. 6 also shows that in the concentration range from 0 to 150 \(\mu\)M, N-oleoyl ethanolamine fails to release Mg\(^{2+}\) from heart mitochondria, even in the presence of added Ca\(^{2+}\). Further increases in the concentration of the compound to as high as 400 \(\mu\)M also failed to induce the release of Mg\(^{2+}\) from the heart organelles (data not shown).

Further differences between the actions of N-oleoyl ethanolamine on liver and heart mitochondria are apparent from its effects on membrane potential and capacity for electron transport. Fig. 7 A shows that N-oleoyl ethanolamine in the absence of Ca\(^{2+}\), produces a decrease in membrane potential of liver mitochondria as determined by the safranin technique. This effect does not require Ca\(^{2+}\), even at low levels, since the same results are obtained in the presence of EGTA (data not shown). Thus, this effect of N-oleoyl ethanolamine is distinguished from the Mg\(^{2+}\) depletion seen in Fig. 6 and does not depend on altered inner membrane permeability. Fig. 7B shows that the maximal decline in membrane potential is a linear function of N-oleoyl ethanolamine concentration for liver mitochondria and that the compound also reduces membrane potential in heart mitochondria, although substantially higher levels are required to produce a given level of depolarization and no effect is seen below approximately 60 \(\mu\)M.

Parallel effects of the compound are seen on the maximal capacities for succinate oxidation. In liver mitochondria, increasing concentrations of N-oleoyl ethanolamine produce an inhibition of uncoupler-stimulated respiration, with a half-maximal effect occurring at approximately 30 \(\mu\)M. Analogous effects are seen in heart mitochondria except that half-maximal inhibition occurs at a 4-fold higher concentration of the inhibitor (Fig. 8). State 4 respiration was only slightly affected by N-oleoyl ethanolamine, increasing by less than 50% at the highest concentrations tested for both types of mitochondria (data not shown). Inhibition of uncoupler-stimulated succinate oxidation does not arise from competitive inhibition of succinate transport or succinate dehydrogenase as shown by the fact that the extent of inhibition at a constant inhibitor concentration is invariant over a wide range of succinate concentrations for mitochondria from both tissues.

Hydrolysis of N-Oleoyl ethanolamine by Mitochondria and Tissue Homogenates—The data presented above show both similarities and differences between the actions of N-oleoyal-

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**Fig. 6.** The effect of N-oleoyl ethanolamine (NAE) concentration on the release of endogenous Mg\(^{2+}\) from Ca\(^{2+}\)-loaded mitochondria. The experiments were performed in parallel to those presented in Fig. 5. Eight minutes after Ca\(^{2+}\) addition, samples were taken for the determination of mitochondrial Mg\(^{2+}\) content as described under "Materials and Methods." ○, liver mitochondria; ●, heart mitochondria.
ethanolamine on heart and liver mitochondria. Apart from the variations in concentration dependence, the major differences relate to the ability of low concentrations of N-oleoylethanolamine together with Ca\textsuperscript{2+} to promote increased permeability of liver but not heart mitochondria (see "Discussion"). Since free fatty acid potentially liberated by hydrolysis of the N-acyl linkage could affect the stability of mitochondria to Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-releasing agents (10), the stability of N-oleoylethanolamine during incubation with these mitochondrial preparations was examined. Fig. 9 shows that the liver preparation does hydrolyze the amide, whereas no hydrolysis by heart mitochondria could be detected. At a nominal N-oleoylethanolamine concentration of 80 \muM, hydrolysis by liver mitochondria was initially linear at a rate of 1.5 nmol/min/mg of protein which then decreased as the reaction proceeded.

Table I shows that except in the case of heart, total homogenates of all tissues tested showed some activity for the hydrolysis of N-oleoylethanolamine. The level of activity in liver homogenates is substantially higher than that present in the other preparations. Heart homogenates fail to hydrolyze the compound at either neutral or acid pH, indicating that the putative hydrolase is not a latent lysosomal enzyme in this tissue.

**DISCUSSION**

The data presented here demonstrate that long chain acylamides of ethanolamine have pronounced effects on several functional parameters of heart and liver mitochondria. In heart mitochondria, these compounds inhibit the release of accumulated Ca\textsuperscript{2+} induced by oxalacetate, N-ethylmaleimide, and palmityl coenzyme A, which are representative of Ca\textsuperscript{2+}-releasing agents that act by promoting increased permeability of the inner mitochondrial membrane (11, 12). This effect of the compound occurs in the concentration range between 0 and approximately 100 \muM. At higher concentrations, N-acylethanolamines also severely diminish the development of membrane potential, reduce the uncoupler-stimulated rate of succinate oxidation, and inhibit energy-dependent Ca\textsuperscript{2+} uptake. These latter effects require an approximately 5-fold higher concentration of the compound to produce a maximal effect, compared to the concentration required to inhibit Ca\textsuperscript{2+} release. Thus, N-acylethanolamines appear to have multiple sites of action. In addition, the concentration dependencies point to potential physiological significance since this lipid class has been found at levels as high as 500 nmol/g of tissue in infarcted canine myocardium (19, 20).

Since the extent of inhibition of uncoupler-stimulated respiration is independent of succinate concentration, this effect of the compound probably arises at the level of the electron transport chain rather than through an inhibition of the dicarboxylate antiporter or of succinate dehydrogenase. Maximal rates of Ca\textsuperscript{2+} uptake can be limited by maximal rates of electron transport (34-36) and the degree to which mitochondria can lower the medium Ca\textsuperscript{2+} concentration is a function of

![Graph](image-url)
the magnitude of the membrane potential at lower values of this parameter (37). The effects of N-acyethanolamine on the rate and extent of Ca$^2+$ uptake by heart mitochondria then appear to be reflections of inhibited electron transport and diminished membrane potential rather than direct effects on the Ca$^2+$ unipporter per se.

The basis for the reduction of membrane potential produced by N-acyethanolamine is less obvious. The concentration dependence for this activity is very similar to the concentration dependence for inhibition of uncoupler-stimulated respiration. However, only the state 4 component of the potential electron transport rate is required to maintain a fully developed membrane potential under normal conditions. In addition, state 4 respiration rates are only slightly elevated by the concentrations of N-acyethanolamines employed in this study, indicating that the compound by itself does not markedly alter the proton conductance of the inner membrane. Thus, one might expect that increasing concentrations of N-acyethanolamine would not diminish membrane potential until the maximal rate of electron transport was reduced to the state 4 region, although this is not observed. It is possible that the loss of membrane potential produced by N-acyethanolamine is accompanied by an increase in the transmembrane pH gradient with the result that the total electrochemical proton gradient remains high. It is not clear, however, from the structure of the compound or its other effects how an increase in ΔpH would occur.

N-Acylethanolamine can inhibit electron transport, the development of membrane potential and Ca$^2+$ transport in liver mitochondria and so these actions in the two mitochondrial types are similar. A given degree of inhibition of these activities requires approximately one-third the level of the inhibitor needed to produce an equivalent effect in heart mitochondria. The reason for the higher sensitivity of the liver organelles is not clear, but may relate to the fact that liver compared to heart mitochondria contain approximately one-half as much phospholipid on a protein basis (32). The effects of N-acyethanolamines on Ca$^2+$ and Ca$^{4+}$-releasing agent-dependent permeability changes in liver mitochondria at first appear to be fundamentally different from those in the heart organelles. Lower levels of the compound are seen to stimulate the rate of swelling induced by Ca$^2+$ plus Ca$^{4+}$-releasing agents, to promote the premature release of accumulated Ca$^{4+}$, and to induce the release of endogenous Mg$^{2+}$ by a mechanism that requires exogenous Ca$^{4+}$. Thus, rather than inhibiting the development of increased permeability produced by Ca$^2+$ and Ca$^{4+}$-releasing agents, at low levels N-acyethanolamine appears to act as a Ca$^{4+}$-releasing agent in liver but not in heart mitochondria. This apparent discrepancy is complicated further by the fact that in liver mitochondria higher levels of N-acyethanolamine result in inhibition of the increased permeability produced by the lower levels.

The differing phenomena observed in mitochondria from the two tissue types can be reconciled when interpreted in view of the fact that liver but not heart mitochondria hydrolyze N-acyethanolamine. Low levels of the compound would therefore be ineffective in partially inhibiting the development of increased permeability in liver mitochondria since they are degraded to a substantial extent during the experiment. The fatty acid produced by the hydrolysis would be expected to lead to the synthesis of intramitochondrial acylcoenzyme A since free fatty acids are weak uncouplers and therefore relatively permeant to the inner membrane (38, 39) and since the intramitochondrial acylcoenzyme A synthetase in liver mitochondria is relatively active toward long chain free fatty acids (40, 41). We showed previously that free fatty acids at levels as low as 1 nmol/mg of protein can markedly increase the sensitivity of mitochondria to Ca$^2+$ plus Ca$^{4+}$-releasing agents through the actions of the acylcoenzyme A derived in this manner (10). Thus, apart from the lack of partial protection by low levels of N-acyethanolamine resulting from its hydrolysis, a product of the hydrolysis leads to accumulation of another Ca$^{4+}$-releasing agent, thereby enhancing the actions of oxalacetate or phosphate as shown in Fig. 4 or independently causing increased permeability of Ca$^{4+}$-loaded mitochondria as seen in Figs. 5 and 6. In agreement with these interpretations, we found that the ability of low levels of N-acyethanolamine to sensitize liver mitochondria to other Ca$^{4+}$-releasing agents or to independently promote increased permeability is antagonized by the presence of carnitine in the media (data not shown). This metabolite can reduce the accumulation of acylcoenzyme A through the action of the intramitochondrial carnitine acyltransferase activities and was shown previously to prevent the sensitizing of liver mitochondria produced by added free fatty acid (10). As the concentration of N-acyethanolamine is increased further, the level present would remain high during the experiment and so protection against increased permeability is observed. This includes protection against the effects of acylcoenzyme A arising from liberated free fatty acid, explaining why high concentrations of the inhibitor eliminate the effects of low concentrations. None of this complexity is seen in heart mitochondria since their ability to hydrolyze N-acyethanolamine is low or absent (Fig. 9) and since the intramitochondrial acylcoenzyme A synthetase in these mitochondria has little activity toward long chain fatty acids (42).

Some further explanation is required regarding the concentration dependencies of N-oleoylthanolamine effects seen in Figs. 4 to 6. Fig. 4 indicates that concentrations of the compound above 15 μM begin to inhibit the development of increased permeability as monitored by swelling, whereas Fig. 6 shows that a 30 to 40 μM concentration is required when permeability is monitored by Mg$^{2+}$ release. In addition, the concentration dependency in Fig. 4 is hyperbolic while some sigmoidal character is apparent in Fig. 6. These variations reflect the fact that the basic phenomenon being observed in Fig. 4 is the transmembrane uptake of mannitol and sucrose driven by the Donnan potential, whereas in Fig. 6, it is the transmembrane diffusion of a divalent cation down its concentration gradient. Since it is reasonable to assume that the diffusion mechanisms and associated energy barriers by which cations and sugar molecules cross the inner membrane would be different, the fact that the concentration dependencies for alterations in the permeability of these species by N-oleoylthanolamine are nonequivalent should not be considered a discrepancy.

It is seen further, comparing Figs. 5 and 6, that concentrations of N-oleoylthanolamine that induce a marked release of Mg$^{2+}$ from Ca$^{4+}$-loaded mitochondria, do not appear to induce an equivalent release of Ca$^{4+}$, at least during the early period following Ca$^{4+}$ uptake. This apparent discrepancy reflects the fact that the development of increased permeability in liver mitochondria treated with Ca$^{4+}$ and a Ca$^{4+}$-releasing agent occurs in a heterogeneous manner. Prior to completion of the process, the suspensions contain a disrupted population which has lost Ca$^{4+}$ and Mg$^{2+}$, together with an intact population still capable of energy-dependent processes. When low levels of phosphate are present as in these experiments, Ca$^{4+}$ released from the initially disrupted mitochondria can be temporarily reaccumulated by surviving mitochondria, whereas Mg$^{2+}$ cannot. Thus, the net release of Ca$^{4+}$ can appear...
to lag behind the release of Mg$^{2+}$ and the effects of low concentrations of N-oleylethanolamine are more readily observed on Mg$^{2+}$ release. As seen in Fig. 1, this situation is less evident in heart mitochondria, presumably because they are capable of energy-dependent Mg$^{2+}$ as well as Ca$^{2+}$ uptake (see Ref. 43 for review).

At concentrations of N-oleylethanolamine between 60 and 100 μM, the development of increased permeability becomes strongly inhibited, while net Ca$^{2+}$ release during the early period following Ca$^{2+}$ uptake becomes apparent. This is consistent with the above discussion since, in this region, maximal rates of electron transport and the development of membrane potential become substantially inhibited. These effects would act to diminish reaccumulation of Ca$^{2+}$ by surviving mitochondria and so allow the release from disrupted mitochondria to be observed. The rate of this release is finally eliminated at the highest concentrations, as the development of increased permeability becomes fully inhibited.

The site where N-acyl ethanolamine acts to inhibit the development of increased inner membrane permeability is a matter of considerable interest. Since it is relatively hydrophobic, yet retains some water solubility, it should be able to enter all compartments in the mitochondrial structure. We have proposed that Ca$^{2+}$ plus Ca$^{2+}$-releasing agents promote the development of increased inner membrane permeability by resulting in the simultaneous activation of intramitochondrial phospholipase A$_{2}$ and inhibition of acylcoenzyme A-lysophospholipid acyltransferase activity, thereby promoting an increase in the inner membrane content of lysophospholipid and free fatty acid. These degradation products are proposed to be concentrated in microdomains where they increase local permeability through physical effects. Analysis of lysophospholipids and free fatty acids and the effects of phospholipase A$_{2}$ inhibitors are consistent with this view (9–13). In the framework of this hypothesis, N-acyl ethanolamine could act by inhibiting phospholipase A$_{2}$ by dispersing microdomains of phospholipase A$_{2}$ reaction products, or by permitting a bypass of the inhibited acyltransferase. No choice among these alternatives is possible from the present data.

An alternative hypothesis maintains that the inner membrane contains an integral component which can produce a pore upon binding Ca$^{2+}$ and thereby allow low and moderate molecular weight compounds to diffuse in and out of mitochondria (44, 45). By this hypothesis, one could propose that N-acyl ethanolamine interferes with Ca$^{2+}$ binding or with the conformational changes necessary to form the pore. Further studies of N-acyl ethanolamine effects on mitochondria may help clarify which permeability control hypothesis is correct.

The present data strengthen and extend the hypothesis that N-acyl ethanolamine acts to inhibit the development of increased inner membrane permeability as a defense against cellular degradation and death. It is increasingly apparent that the degree to which cellular and subcellular membrane integrity can be maintained is a critical determinant of the reversibility of cellular injury, including injury by ischemia. It appears that the elevation of intracellular Ca$^{2+}$ concentrations to the millimolar range is the primary insult produced by a variety of agents or conditions which cause cell injury (46), that elevated Ca$^{2+}$ is a primary cause of membrane degradation and that Ca$^{2+}$-activated phospholipases are involved in this process (47–50). Several recent studies have implicated the survival of mitochondrial functions (dependent on the maintenance of an impermeable inner membrane) as pivotal in the recovery from episodes of ischemia (16, 18, 51, 52). This background information, together with the known requirements for the biosynthesis of N-acyl ethanolamine and the data presented here, identifies this class of compounds as ideal candidates for protective agents.

Synthesis of N-acyl ethanolamine requires Ca$^{2+}$ at millimolar levels (22) and thus could be triggered by the primary insult, elevated Ca$^{2+}$. Lysophosphatidylethanolamine is one of the substrates from which the compound is made (22). This is particularly significant since 80% of the lysophospholipid produced by the inner membrane phospholipase A$_{2}$ during the development of increased permeability is lysophosphatidylethanolamine (13). Thus, production of N-acyl ethanolamine consumes the compound thought to be primarily responsible for increased permeability and while producing a product which antagonizes action of remaining substrate, possibly by inhibiting Ca$^{2+}$-activated phospholipase A$_{2}$. These effects would tend to promote the maintenance of mitochondrial functions which are critical to the eventual recovery of the cell.

The activities of N-acyl ethanolamine as an inhibitor of Ca$^{2+}$ uptake and electron transport can also be construed as potentially beneficial to ischemic tissue. Inhibition of Ca$^{2+}$ uptake would be advantageous since Ca$^{2+}$ must be accumulated into the matrix space to activate the critical phospholipase A$_{2}$. Inhibition of electron transport by the mitochondria of the most severely damaged cells (those containing the highest Ca$^{2+}$ and N-acyl ethanolamine levels and those whose mitochondria are most severely uncoupled) would tend to spare any available oxygen for use by more intact cells (presumably containing less Ca$^{2+}$ and N-acyl ethanolamine and more intact mitochondria) which have a higher probability of eventual recovery.

It is also reasonable to propose that the protective actions of N-acyl ethanolamine may not be confined to the inner mitochondrial membrane or to tissues injured by ischemia. This lipid class may act to stabilize other cellular and subcellular membranes and may be beneficial in tissue injury by any mechanism where massive increases in intracellular Ca$^{2+}$ concentrations are involved. Determining to what degree these compounds are of general significance requires a broader survey of where they occur and a more detailed understanding of their synthesis and degradation.

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Inhibition of permeability-dependent Ca\textsuperscript{2+} release from mitochondria by N-acyl ethanolamines, a class of lipids synthesized in ischemic heart tissue.

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