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Hepatic calcium efflux during cytochrome *P*-450-dependent drug oxidations at the endoplasmic reticulum in intact liver

(NADPH oxidation/perfused rat liver/thiol redox state)

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ABSTRACT **During metabolism of (type I) drugs by cytochrome *P*-450-dependent monooxygenase of the endoplasmic reticulum, the NADPH/$NADP^+$ ratio in rat liver selectively decreases to approximately one-half of the control values, whereas the NADH/NAD^+ ratio remains practically unaffected [Sies, H. & Brauser, B. (1970) *Eur. J. Biochem.* 15, 531–540]. In view of the observations with isolated mitochondria [Lehninger, A. L., Vercesi, A. & Bababunmi, E. A. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1690–1694] of stimulated Ca^{2+} efflux upon nicotinamide nucleotide oxidation, the selective oxidation of NADPH in cytosol and mitochondria during drug oxidations was considered a useful experimental tool for the determination of whether the oxidation of NADPH or of NADH is responsible for Ca^{2+} efflux. With perfused livers from phenobarbital-treated rats, Ca^{2+} efflux was demonstrated, amounting to 8 nmol/min per gram of liver (wet weight), with aminopyrine, ethylmorphine, or hexobarbital as drug substrates. Drug-associated Ca^{2+} release was diminished when the inhibitor metyrapone was also present, or when drug oxidation was suppressed during N_2 anoxia or in the presence of antimycin A in livers from fasted rats. Ca^{2+} efflux was elicited also by infusion of the thiol oxidant diamide, and by *t*-butyl hydroperoxide. However, whereas Ca^{2+} efflux elicited by these compounds was restricted upon addition of the thiol dithioerythritol, there was little, if any, sensitivity of the drug-associated Ca^{2+} efflux to the thiol. Further mitochondrial oxidation of NADPH by addition of ammonium chloride had no effect on drug-associated Ca^{2+} efflux. Prior addition of the α-agonist phenylephrine suppressed the Ca^{2+} release by drug addition. While the molecular mechanism involved in Ca^{2+} efflux from liver mitochondria and from hepatocytes as well as the regulatory significance are not yet known, it is concluded from the present experiments that in case of nicotinamide nucleotide-linked Ca^{2+} efflux the oxidation of NADPH may suffice, with oxidation of NADH not being a requirement.**

Calcium efflux from isolated mitochondria can be stimulated by a variety of metabolic transitions, and the hypothesis was put forward by Lehninger *et al.* (1) that redox transitions are crucial, in particular the oxidation of reduced nicotinamide nucleotides. Evidence consistent with this hypothesis was provided from experiments in which *t*-butyl hydroperoxide was used as oxidant (2), and from a comparison of the oxalacetate response in glucagon-challenged and control mitochondria (3). Because the molecular mechanism of the nicotinamide nucleotide effects is still unknown, a distinction between the oxidation of NADPH and of NADH as the agent primarily involved appears to be of interest. Some evidence pointing towards a preferential role of NADPH is available (3). Ca^{2+} efflux from isolated hepatocytes was also found to be stimulated when mitochondrial nicotinamide nucleotides were oxidized (4) and, conversely, Ca^{2+} addition led to an increased reduction of mitochondrial $NAD(P)^+$ in hepatocytes (5). However, efflux from intact cells may not necessarily be linked to efflux from the mitochondrial matrix space, because a considerable share of cellular Ca^{2+} is associated with the endoplasmic reticulum and with the plasma membrane (6).

It is now well established that during enhanced electron flux through the cytochrome *P*-450-dependent monooxygenase system of the endoplasmic reticulum the cellular NADPH/$NADP^+$ is decreased substantially, whereas NADH/NAD^+ is not significantly affected. For example, in perfused liver NADPH/$NADP^+$ decreased from 4.0 in the controls to 2.3 during oxidation of a drug substrate for cytochrome *P*-450, hexobarbital, with NADH/NAD^+ remaining unchanged (7). This has been confirmed by various groups employing different drug substrates by direct measurement or with redox indicator metabolites both in isolated hepatocytes and in isolated perfused liver (8–11). Further, it is noteworthy that the NADPH oxidation occurs in both the cytosolic and the mitochondrial compartments of the hepatocyte (11).

The selective oxidation in the NADPH system initiated at the endoplasmic reticulum, therefore, seemed a useful tool to investigate redox-linked properties of Ca^{2+} efflux in the intact cell. In the present work, we have examined Ca^{2+} release from the isolated perfused rat liver during drug oxidations.

MATERIALS AND METHODS

Hemoglobin-Free Perfusion of Rat Liver. Livers of male Wistar rats of 130–190 g body weight, fed on stock diet (Altromin), were perfused as described previously without recirculation of the perfusate, using the bicarbonate-buffered Krebs–Henseleit solution except for Ca^{2+} concentrations (12). The Ca^{2+} concentration was 1.25 mM during the initial period of perfusion, approximately 10 min, and then the Ca^{2+} was lowered to 10 μM for another 10–15 min before switching to a medium with 5 μM Ca^{2+}, everything else being constant. In order to increase monooxygenase activity, the animals were pretreated with sodium phenobarbital (1 g/liter of drinking water) for at least 7 days. Perfusate flow (3.5–4.5 ml min^{-1} g of $liver^{-1}$) was maintained constant throughout the individual perfusion experiment. The temperature was 37°C.

Assays. Effluent caval perfusate was monitored continuously for O_2, pH, and pCa^{2+} by using appropriate electrodes (12). The Ca^{2+}-specific electrode was operated as described previously in detail for the K^+ and NH_4^+ electrodes (13). Because small Ca^{2+} activity changes were studied, usually the antilog step was not used; within the calibrated range of 5–10 μM, the trace can be considered linear with Ca^{2+} activity. Assays for the metabolites in the perfusion medium (2.1 mM L-lactate, 0.3 mM pyruvate, 0.5 mM DL-β-hydroxybutyrate, and 0.3 mM acetoacetate) were carried out according to the procedures described in ref. 14.

Apparatus and Chemicals. The Ca^{2+}-specific electrode was generously provided by Philips (Kassel, Fed. Rep. Germany).

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Aminopyrine (Hoechst, Frankfurt), hexobarbital (Bayer, Leverkusen), and metyrapone (CIBA, Basel, Switzerland) were gifts from the manufacturers. Ethylmorphine (Merck, Darmstadt) and antimycin A (Serva, Heidelberg) as well as other chemicals, biochemicals, and enzymes (Merck and Boehringer Mannheim) were obtained commercially.

RESULTS

Calcium Efflux from Perfused Liver During Drug Oxidations. The perfused liver releases Ca^{2+} into the effluent caval perfusate when flux through the NADPH-dependent monooxygenase system with cytochrome P-450 as terminal oxidase is stimulated by the addition of a drug substrate, aminopyrine (Fig. 1). The effect is rapid and reversible. In this particular experiment, the influent perfusate contained 1.25 mM Ca^{2+} until 13 min, then 10 μM Ca^{2+} until 28 min, when the final concentration of 5 μM was given. This way, the electrode trace is calibrated, independent of any trace amounts of Ca^{2+} that might be present in the other salt components of the Krebs–Henseleit solution. Further, it is seen that during this early period in the experiment there is a slight downward drift, due to a slowly decreasing basal Ca^{2+} release. The extra Ca^{2+} efflux upon the addition of aminopyrine is 3 μM, amounting to a rate of 12 nmol min^{-1} g of $liver^{-1}$. Similar observations were obtained when other drug substrates were used—e.g., ethylmorphine or hexobarbital (Table 1).

Dependence of Drug-Induced Calcium Efflux on Monooxygenase Activity. When cytochrome *P*-450-dependent drug oxidation was inhibited by the prior addition of metyrapone, both the extra O_2 uptake and the extra Ca^{2+} efflux upon addition of ethylmorphine (Fig. 2) or of aminopyrine (not shown) were suppressed. Alternatively, when the O_2 concentration in the effluent perfusate was artificially decreased by appropriate O_2/N_2 mixtures in the oxygenator toward the level observed upon addition of drug substrate, there was no extra Ca^{2+} release. Also, there was no extra Ca^{2+} efflux when the drugs were infused during anoxic conditions (replacement of O_2 by N_2).

The oxidation of aminopyrine and ethylmorphine leads to the generation of formaldehyde; formaldehyde (0.4 mM) infusion did not influence the Ca^{2+} concentration in the effluent perfusate. Hexobarbital, a drug that is *C*-hydroxylated rather than *N*-demethylated and thus does not give rise to formaldehyde, provides Ca^{2+} efflux rates similar to those of the former drugs.

Table 1. Calcium efflux from perfused liver during drug oxidations

Nutritional state of rat	Addition: Drug	Addition: Conc., mM	Ca^{2+} efflux, nmol min^{-1} g of $liver^{-1}$
Fed	Aminopyrine	0.86 ± 0.03 (9)	6.2 ± 1.2 (9)
Fed	Ethylmorphine	0.84 ± 0.08 (6)	8.7 ± 1.7 (6)
Fed	Ethylmorphine	1.23 ± 0.08 (5)	12.4 ± 1.6 (5)
Fasted 1 day	Ethylmorphine	1.17 ± 0.10 (4)	8.2 ± 0.5 (4)
Fed	Hexobarbital	0.86 ± 0.10 (4)	9.2 ± 1.8 (4)
Fed	Ammonium chloride	1.0	1.0 – 2.0
Fed	*t*-Butyl hydroperoxide	0.9	17.6

Livers from phenobarbital-pretreated male rats were perfused with Krebs–Henseleit solution containing 5 μM Ca^{2+}. The extra Ca^{2+} efflux upon additon of drug substrates was calculated from the plateau levels observed in effluent Ca^{2+} approximately 2 min after onset of substrate infusion. For ethylmorphine, the values are corrected for a blank value corresponding to about 1 mmol of Ca^{2+} per mol of ethylmorphine, whereas for hexobarbital a slight blank value in the opposite direction had to be corrected for. Data are given as means ± SEM, with the number of different perfusion experiments in parentheses.

The nutritional state slightly affects the Ca^{2+} efflux, which is higher with livers from fed than from fasted rats (Table 1).

Mitochondrial–Cytosolic Interrelationships. Subfractionation experiments with isolated hepatocytes revealed that the free NADPH/$NADP^+$ system undergoes oxidation both in the cytosol and in the mitochondrial matrix space during aminopyrine oxidation, as indicated by a similar response in the isocitrate/2-oxoglutarate couple in both of these subcellular compartments (11). Thus, the possibility exists from these results that the Ca^{2+} efflux observed during drug oxidation is a consequence of a cytosolic or mitochondrial redox transition or both.

In order to further oxidize the mitochondrial matrix NADPH (15), ammonium chloride was infused. However, Ca^{2+} efflux

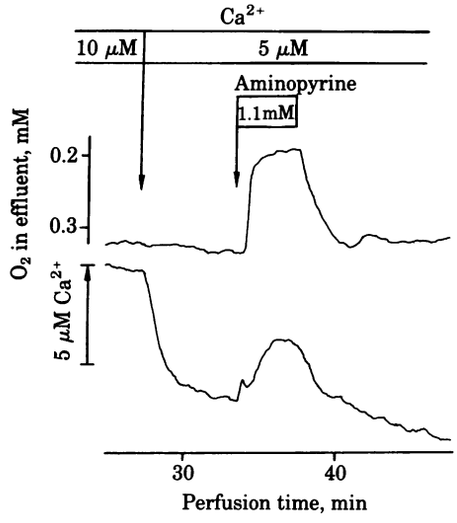

FIG. 1. Ca^{2+} efflux during metabolism of aminopyrine in perfused liver from phenobarbital-pretreated rat. Ca^{2+} (lower traces) and O_2 (upper trace) concentrations in the effluent perfusate collected from the cannulated caval vein were recorded as a function of time. Top shows additions of Ca^{2+} and of aminopyrine to the influent perfusate entering via the portal vein.

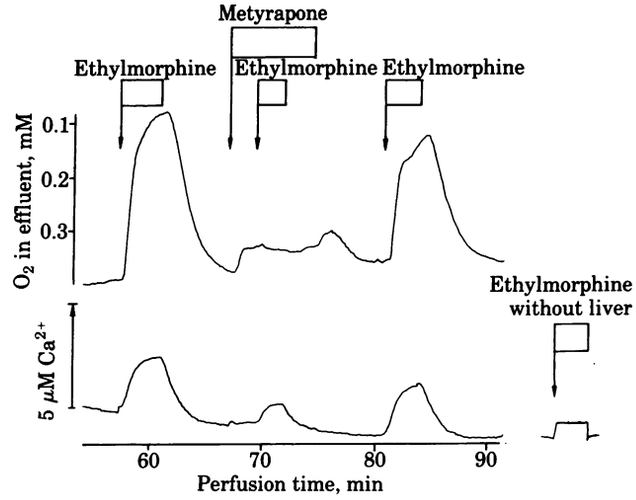

FIG. 2. Suppression of drug-induced Ca^{2+} efflux by an inhibitor of drug metabolism, metyrapone (0.44 mM). Ethylmorphine was infused at 0.9 mM concentration as indicated. The inhibition of drug oxidation by metyrapone is documented by lack of extra O_2 uptake. The small rise in Ca^{2+} trace between 70 and 72 min is due to Ca^{2+} contained in the ethylmorphine sample, as shown in the blank value at right.

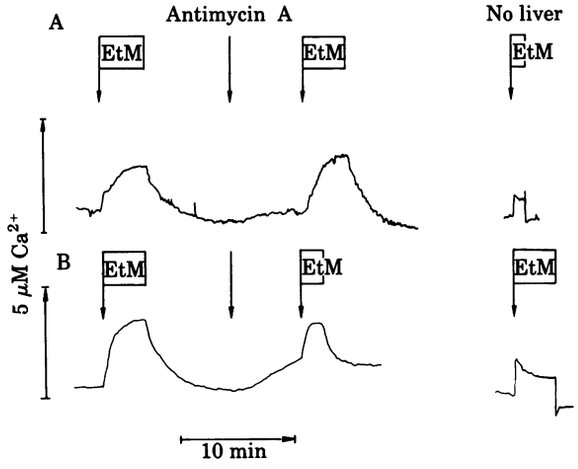

FIG. 3. Effect of antimycin A on ethylmorphine (EtM)-induced Ca^{2+} efflux in perfused liver from fed (*A*) and 24-hr fasted (*B*) rats. Ethylmorphine (*A*, 0.7 mM; *B*, 0.9 mM) and antimycin A (60 nmol/min per g wet weight of liver) were infused as indicated. Note blank value for ethylmorphine shown at right.

was stimulated only slightly by ammonium chloride (about 2.0 nmol min^{-1} g of $liver^{-1}$) in the presence and in the absence of ethylmorphine; this was the case with livers from both fed and fasted animals. On the other hand, when mitochondrial electron flux was blocked by the addition of antimycin A, the Ca^{2+} efflux elicited by ethylmorphine was observed only with livers from fed rats, not from fasted rats (Fig. 3). Due to the presence of glycogen substrate for the pentose phosphate pathway in livers of fed rats, reducing equivalents for drug oxidations can be supplied in spite of the presence of antimycin A in livers from fed rats but not from fasted rats, as is also indicated by a corresponding increase in O_2 uptake upon drug addition in livers from fed rats but not from fasted rats, not shown here (cf. refs. 7 and 16). Therefore, these findings confirm the results obtained above with metyrapone, namely that the Ca^{2+} efflux upon drug addition is dependent on flux through the cytochrome *P*-450-dependent monooxygenase system.

Relationship to Phenylephrine-Mobilizable Ca^{2+} Pool. Recently, it was concluded from experiments with perfused liver that the mobilizable Ca^{2+} pool responsive to addition of the α-adrenergic agonist phenylephrine is associated with the plasma membrane (17). However, similar experiments with perfused liver (18) and with isolated hepatocytes (19, 20) provided evidence for a mitochondrial localization of the responsive pool and a redistribution in favor of the microsomal compartment (20). The experiment in Fig. 4 shows that the Ca^{2+} pool mobilizable from the liver by aminopyrine is included in the (much larger) Ca^{2+} pool mobilizable by the α-agonist. The aminopyrine-elicited Ca^{2+} efflux is absent when phenylephrine has been given prior to the addition of the drug (Fig. 4*A*). However, after replenishment of the hepatic Ca^{2+} stores from extracellular Ca^{2+} provided by a perfusion interval (30 min) with high Ca^{2+} (1.25 mM), the aminopyrine response is demonstrable with the same liver (Fig. 4*B*).

Relationship to Thiol Redox State. The oxidative transitions elicited by the additions shown in Table 1 all are associated, to various degrees, with an oxidative transition in the glutathione

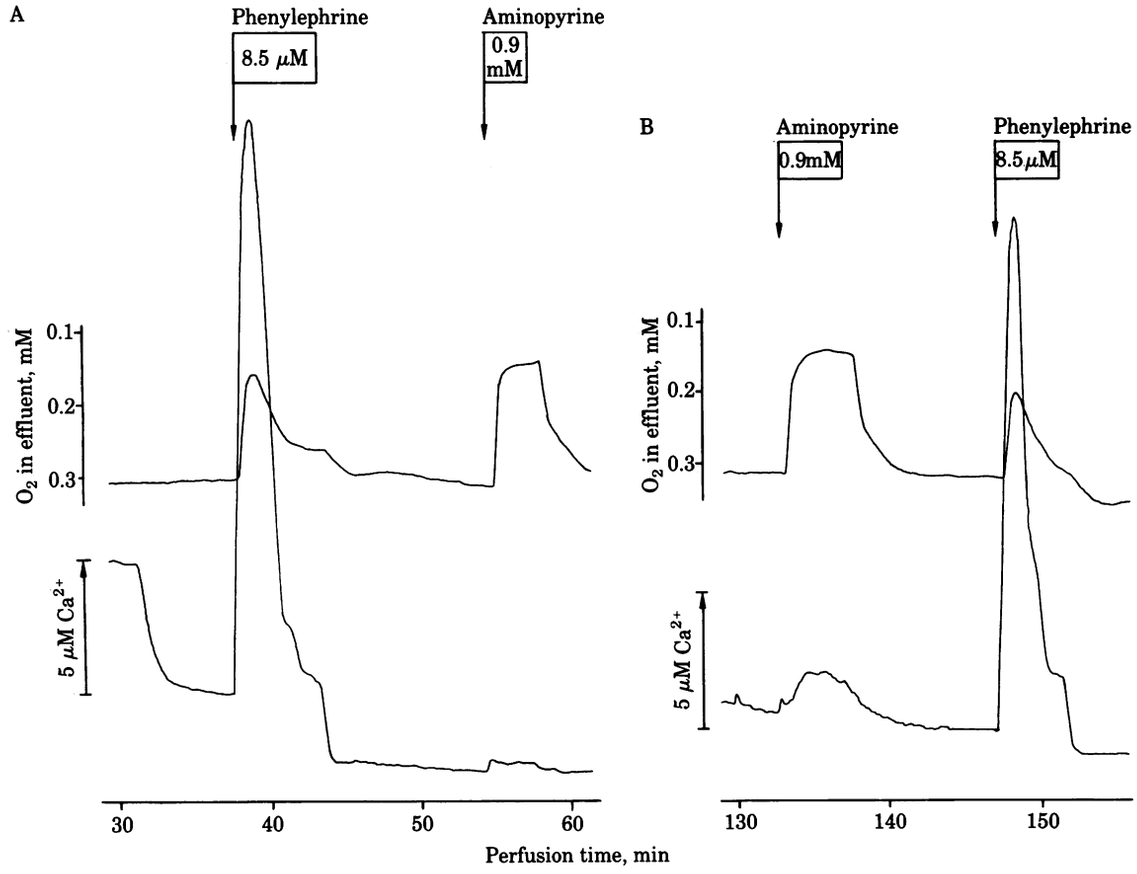

FIG. 4. Phenylephrine and aminopyrine effects on Ca^{2+} efflux. The prior addition of phenylephrine abolishes the aminopyrine response (*A*). However, in the same experiment the aminopyrine-releasable Ca^{2+} pool was replenished by perfusing with high Ca^{2+} (1.25 mM) for 30 min before returning to the condition in *A*. Order of additions was then reversed (*B*).

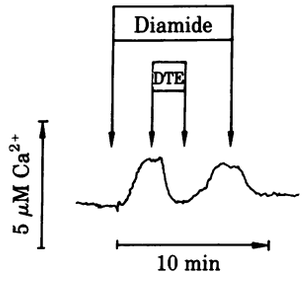

FIG. 5. Ca^{2+} efflux from perfused rat liver upon addition of diamide (0.7 mM) and its reversal by dithioerythritol (DTE; 1.4 mM).

system: *t*-butyl hydroperoxide is reduced to *t*-butyl alcohol by glutathione peroxidase, yielding oxidized glutathione (GSSG) directly (21, 22), whereas GSSG efflux observed during oxidations by cytochrome *P*-450 may arise from the formation of H_2O_2 or organic hydroperoxides accompanying these drug oxidations (23, 24). Therefore, and because of known effects of thiol-specific reagents on Ca^{2+} efflux from isolated mitochondria (25), we tested the question whether the Ca^{2+} efflux from perfused liver during drug oxidations may be related to the thiol redox state. In fact, an extra Ca^{2+} efflux was initiated upon the addition of diamide, the thiol oxidant, and this effect was abolished upon the additional infusion of the thiol reductant dithioerythritol (Fig. 5), indicating that an oxidative transition in the thiol redox state is capable of releasing Ca^{2+} also from the intact organ. However, when dithioerythritol was added during drug oxidation, e.g., of ethylmorphine, there was little change; also, the prior addition of dithioerythritol had no significant influence on the extra Ca^{2+} release elicited by ethylmorphine (not shown). Thus, for drug-associated Ca^{2+} release to occur, bulk phase thiol oxidation does not appear to be a requirement. Furthermore, the depletion of cellular glutathione levels by treatment of the animals with a glutathione-alkylating electrophile, diethylmaleate, at a dose of 1.2 mmol/kg of body weight 1 hr prior to the experiment had no effect on Ca^{2+} efflux: the results were virtually superposable on those of controls—e.g., Fig. 2.

DISCUSSION

The experiments reported here demonstrate that an efflux of Ca^{2+} from the liver cell into the extracellular space is triggered upon enhancement of electron flux through the cytochrome *P*-450-dependent monooxygenase system by the addition of drug substrates. Ca^{2+} efflux was restricted when drug substrates were added under conditions not permitting an increased electron flux—e.g., with metyrapone, under anoxic conditions, or in the presence of antimycin A in livers of fasted rats. The employed experimental setup, therefore, allows the conclusion that the observed Ca^{2+} efflux depends, as a primary event, on the oxidation of NADPH at the endoplasmic reticulum. Although synergistic effects of NADPH and NADH have been described for drug oxidations in isolated microsomes (26), net oxidation in the NADH system does not occur in the intact cells (7–11). In fact, there is a slight increase in the lactate-to-pyruvate and hydroxybutyrate-to-acetoacetate ratios during aminopyrine metabolism (27), whereas the isocitrate-to-2-oxoglutarate ratio decreases to one-half of the control (11). Thus, the nicotinamide nucleotide oxidized is selectively NADPH.

Ca^{2+} efflux from the cell may result simply from the outer phase of the plasma membrane (17). However, under the condition of artificially low extracellular Ca^{2+} used here, fluctuations in cytosolic free Ca^{2+} may be transmitted that will not occur at the high physiological plasma Ca^{2+} concentration. The experimental setup is, therefore, particularly suited to also indicate input from membrane-bound Ca^{2+} to the cytosol from mitochondria, endoplasmic reticulum, or both. The plasma membrane, in fact, was recently considered (28) unlikely to be a primary determinant of cytosolic free Ca^{2+} because the free Ca^{2+} concentration of 0.2 μM maintained by mixtures of isolated mitochondria and microsomes (28) was similar to that of isolated hepatocytes (20).

The molecular mechanism of the facilitation of Ca^{2+} release remains to be elucidated. For example, a process such as ADP-ribosylation by nicotinamide nucleotides (29) or a role of the extramitochondrial pH (30) have been proposed. In a recent report, the measured ratios of NADPH/$NADP^+$ correlated with Ca^{2+} efflux from isolated mitochondria under various conditions, whereas NADH/NAD^+ ratios did not (31), but this distinction between $NADP^+$ and NAD^+ systems was not discussed by the authors of (31). Except for a lack of a stimulatory effect of added ammonium ions, known to selectively oxidize mitochondrial matrix NADPH (15), the observations on the whole liver presented here are compatible with previous results obtained with isolated mitochondria (1–3, 29, 31). However, as stated above, at present we cannot draw a conclusion on the subcellular location of the Ca^{2+} pool responsive during drug oxidations. The possible physiological significance of nicotinamide nucleotide-dependent Ca^{2+} movements has recently been questioned because of a collapse of the membrane potential (32), but in similar experiments the membrane potential remained high, indicating structural and functional integrity during conditions of facilitated Ca^{2+} efflux (29).

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1. Lehninger, A. L., Vercesi, A. & Bababunmi, E. A. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1690–1694.
2. Lötscher, H.-R., Winterhalter, K. H., Carafoli, E. & Richter, C. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4340–4344.
3. Prpić, V. & Bygrave, F. L. (1980) *J. Biol. Chem.* **255**, 6193–6199.
4. Krell, H., Baur, H. & Pfaff, E. (1979) *Eur. J. Biochem.* **101**, 349–364.
5. Ontko, J. A., Otto, D. A., Oshino, N. & Chance, B. (1975) *FEBS Lett.* **53**, 297–301.
6. Claret-Berthon, B., Claret, M. & Mazet, J. A. (1977) *J. Physiol. (London)* **272**, 529–552.
7. Sies, H. & Brauser, B. (1970) *Eur. J. Biochem.* **15**, 531–540.
8. Sies, H. & Kandel, M. (1970) *FEBS Lett.* **9**, 205–208.
9. Moldéus, P., Grundin, R., Vadi, H. & Orrenius, S. (1974) *Eur. J. Biochem.* **46**, 351–360.
10. Kauffman, F. C., Evans, R. K. & Thurman, R. G. (1977) *Biochem. J.* **166**, 583–592.
11. Weigl, K. & Sies, H. (1978) *Eur. J. Biochem.* **77**, 401–408.
12. Sies, H. (1978) *Methods Enzymol.* **52**, 48–59.
13. Sies, H. (1976) in *Ion and Enzyme Electrodes in Biology and Medicine*, eds. Kessler, M., Clark, L. C., Lübbers, D. W., Silver, I. A. & Simon, W. (Urban & Schwarzenberg, Berlin), pp. 261–267.
14. Bergmeyer, H. U., ed. (1974) *Methoden der enzymatishen Analyse* (Chemie, Weinheim, Fed. Rep. Germany), 3rd Ed.
15. Sies, H., Akerboom, T. P. M. & Tager, J. M. (1977) *Eur. J. Biochem.* **72**, 301–307.
16. Thurman, R. G. & Scholz, R. (1969) *Eur. J. Biochem.* **10**, 459–467.
17. Althaus-Salzmann, M., Carafoli, E. & Jakob, A. (1980) *Eur. J. Biochem.* **106**, 241–248.
18. Blackmore, P. F., Dehaye, J.-P. & Exton, J. H. (1979) *J. Biol. Chem.* **254**, 6945–6950.
19. Babcock, D. F., Chen, J.-L. J., Yip, B. P. & Lardy, H. A. (1979) *J. Biol. Chem.* **254**, 8117–8120.
20. Murphy, E., Coll, K., Rich, T. L. & Williamson, J. R. (1980) *J. Biol. Chem.* **255**, 6600–6608.
21. Sies, H., Gerstenecker, C., Menzel, H. & Flohé, L. (1972) *FEBS Lett.* **27**, 171–175.
22. Oshino, N. & Chance, B. (1977) *Biochem. J.* **162**, 509–525.

23. Sies, H., Bartoli, G. M., Burk, R. F. & Waydhas, C. (1978) *Eur. J. Biochem.* **89,** 113–118.
24. Jones, D. P., Thor, H., Andersson, B. & Orrenius, S. (1978) *J. Biol. Chem.* **253,** 6031–6037.
25. Harris, E. J., Al-Shaikhaly, M. & Baum, H. (1979) *Biochem. J.* **192,** 455–464.
26. Cohen, B. S. & Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143,** 46–53.
27. Waydhas, C., Weigl, K. & Sies, H. (1978) *Eur. J. Biochem.* **89,** 143–150.
28. Becker, G. L., Fiskum, G. & Lehninger, A. L. (1980) *J. Biol. Chem.* **255,** 9009–9012.
29. Lötscher, H.-R., Winterhalter, K. H., Carafoli, E. & Richter, C. (1980) *J. Biol. Chem.* **255,** 9325–9330.
30. Tsokos, J., Cornwell, T. F. & Vlasuk, G. (1980) *FEBS Lett.* **119,** 297–300.
31. Wolkowicz, P. E. & McMillin-Wood, J. (1980) *J. Biol. Chem.* **255,** 10348–10353.
32. Nicholls, D. G. & Brand, M. D. (1980) *Biochem. J.* **188,** 113–118.