O2, in Table 1, since mitochondrial superoxide production increases linearly with O2 tension [8]. Whereas O2 had no effect on \( \Delta \psi \) development at low Ca\(^{2+} \), \( \Delta \psi \) dissipation at high Ca\(^{2+} \) was markedly potentiated. Thus oxidative stress acted in strict synergism with Ca\(^{2+} \) (as observed previously with hydroperoxides [5]).

The adverse effects of Ca\(^{2+} \) and oxidative stress were abolished by 5 mM-ATP (which was maintained by 40 mM-creatine phosphate plus 20 units of creatinephosphokinase), but lower concentrations of ATP were only partially effective. A supraphysiological concentration of ADP (maintained with 10 units of hexokinase plus 50 mM-glucose) on the other hand afforded little protection.

It may be concluded that the pathophysiological free Ca\(^{2+} \) concentration likely to be encountered after prolonged ischaemia (e.g. [9]), might well induce inner membrane pore opening when accompanied by high P, concentration and oxidative stress, provided that cellular ATP is substantially depleted. A prolonged period of ischaemia may be necessary, depending on the tissue, before this critical condition is attained. In heart, irreversible injury is generally associated with a decline of cellular ATP to <40% of normoxic values [10], which may be rapidly followed by rise in cytosolic Ca\(^{2+} \) beyond the 2 \( \mu \)M threshold [9]. Since a high cytosolic phosphorylation potential is required for maintenance of low cytosolic Ca\(^{2+} \), any impairment of oxidative phosphorylation by Ca\(^{2+} \)-induced pore opening might well initiate a vicious cycle of irreversible injury [4, 6].

Table 1 also shows that even under the worst scenario with high Ca\(^{2+} \) concentration, high P, concentration, oxidative stress and without added ATP, 0.6 \( \mu \)M-cyclosporin allowed full development of \( \Delta \psi \). Thus cyclosporin may be of therapeutic value in halting the progression to irreversible injury during reperfusion. Identification of the molecular target of cyclosporin may also help to resolve the protein components involved in pore opening.


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Cyclosporin and mitochondrial phospholipid degradation

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It is generally agreed that cellular Ca\(^{2+} \) overload, oxidative stress and increased inorganic phosphate (P,) are important factors in the injury associated with reperfusion of ischaemic tissue [1]. It has often been suggested that mitochondrial dysfunction induced by excessive Ca\(^{2+} \) uptake may contribute to the progression of the injury; in particular, reperfusion with the inhibitor of mitochondrial Ca\(^{2+} \) uptake, Ruthenium Red (which appears to enter heart cells, [2]), improves mitochondrial function [3] and myocardial contractility [4]. It has been proposed that the mitochondrial lesion is a non-specific pore of about 20 Å diameter that opens under the synergistic influence of high intramitochondrial Ca\(^{2+} \), increased P, and oxidative stress [5, 6]; opening of the putative pore is blocked by low concentrations of cyclosporin [7].

Exposure of liver mitochondria to a high (unbuffered) Ca\(^{2+} \) concentration leads to activation of phospholipase A; [8], and the question arises whether non-specific leakiness caused by hydrolysis of inner membrane phospholipids may contribute to Ca\(^{2+} \)-induced permeabilization [9] or whether the phenomenon and its inhibition by cyclosporin is wholly attributable to a protein pore structure [5]. This study examines this question using Ca\(^{2+} \)-buffered conditions that more closely mimic those of cellular Ca\(^{2+} \) overload [10]. About 42% of the fatty acids esterified at C-2 of mitochondrial phospholipids are polyunsaturated [8], and phospholipase A, action would be expected to increase free, in particular polyunsaturated, fatty acids. In Table 1, respir-

Table 1. Effects of Ca\(^{2+} \) on mitochondrial inner membrane potential and phospholipid hydrolysis

<table>
<thead>
<tr>
<th>[Ca(^{2+} )]/( \mu )M</th>
<th>Cyclosporin (1 ( \mu )M)</th>
<th>( \Delta \psi ) (mV)</th>
<th>LysoPL (%)</th>
<th>Total FFA (( \mu )g/mg of protein)</th>
<th>Polyunsaturated FFA (% of total FFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>–</td>
<td>171</td>
<td>8.9</td>
<td>71</td>
<td>53</td>
</tr>
<tr>
<td>5.0</td>
<td>–</td>
<td>&lt;50</td>
<td>6.7</td>
<td>61</td>
<td>50</td>
</tr>
<tr>
<td>0.3</td>
<td>+</td>
<td>170</td>
<td>8.7</td>
<td>60</td>
<td>51</td>
</tr>
<tr>
<td>5.0</td>
<td>+</td>
<td>168</td>
<td>7.6</td>
<td>65</td>
<td>52</td>
</tr>
</tbody>
</table>

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ing mitochondria were exposed to buffered Ca\(^{2+}\) concentrations that were insufficient (0.3 \(\mu M\)) and sufficient (5 \(\mu M\)) to induce pore opening and uncoupling as shown by the dissipation of the inner membrane potential (\(\Delta$$\psi$$\)). The depolarization was not associated with increase in either total free fatty acids or free polyunsaturated fatty acids (18:2, 20:4, 22:6). In three such experiments, any Ca\(^{2+}\)-induced increase in polyunsaturated free fatty acids was quite undetectable (2 \(\mu g/mg\) of protein \(\pm 6\); mean \(\pm S.E.M.\)). Similarly, no increase was observed in lysophospholipids derived from the major phospholipids, phosphatidylcholine and phosphatidylethanolamine. Moreover, whereas cyclosporin completely prevented Ca\(^{2+}\)-induced depolarization of the inner membrane, no indication was obtained that cyclosporin affected phospholipid metabolism.

It may be concluded that permeabilization under Ca\(^{2+}\)-buffered conditions, and its inhibition by cyclosporin [10], probably involves the reversible opening of a protein pore rather than perturbation of the lipid bilayer. According to the pore hypothesis [5, 7], pore-mediated mitochondrial uncoupling on reperfusion may impair generation of a high cytoplasmic phosphorylation potential required for maintenance of low cytoplasmic Ca\(^{2+}\), allowing further Ca\(^{2+}\) entry into the cell, further pore opening, and so on, so that the cell enters a vicious cycle of irreversible injury. If the hypothesis is correct, cyclosporin may offer therapeutic potential in preventing injury.


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Separation of pre- and post-synaptic receptors on Percoll gradients

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It is increasingly recognized that nicotinic acetylcholine receptors (nAChR) modulate the release of neurotransmitters in the brain, and this is a reflection of their pre-synaptic localization on nerve terminals [1]. However, the relative abundance of presynaptic nAChR in the brain is not known. Functional studies have also shown that muscarinic receptors are present on both pre- and post-synaptic membranes. However, it has proved difficult to distinguish pre- and post-synaptic receptors in binding assays because (i) ligands do not discriminate between them, and (ii) pre- and post-synaptic membranes have not been reliably separated during tissue fractionation studies.

A novel subcellular fractionation technique in which rat brain S1 fraction is applied to four-step Percoll gradients [2] appears to resolve pre- and post-synaptic elements. This non-equilibrium procedure separates particles on the basis of size and density; thus light membrane fragments remain in the uppermost gradient fractions, and account for the majority of postsynaptic muscarinic and nicotinic radioligand-binding sites (see Table 1).

Acetylcholinesterase (AChE; EC 3.1.1.7) activity was broadly distributed across the gradient, reflecting its widespread association with neuronal plasma membranes. Choline acetyltransferase (ChAT; EC 2.3.1.6), on the other hand, predominated in fraction 4, with little activity in the uppermost gradient fractions. This is consistent with the localization of ChAT in hippocampal nerve terminals [3], which are recovered in fractions 3 and 4. Indeed, this is confirmed by the distribution of [\(3^H\)]nicotine binding to Percoll itself. This marker of functional cholinergic terminals is not bound to subsequently recovered postsynaptic ChAT activity [4]. The recovery of 25% of ChAT activity in the mitochondrial fraction, fraction 5, compared with only 6% for nicotine uptake, may reflect the presence of non-viable synaptosomes in this fraction [5].

The muscarinic and nicotinic ligand-binding sites displayed quite different distributions. [\(3^H\)]Hippocampal nerve terminals [3], which are recovered in fractions 3 and 4. Indeed, this is confirmed by the distribution of [\(3^H\)]nicotine binding to Percoll itself. This marker of functional cholinergic terminals is not bound to subsequently recovered postsynaptic ChAT activity [4]. The recovery of 25% of ChAT activity in the mitochondrial fraction, fraction 5, compared with only 6% for nicotine uptake, may reflect the presence of non-viable synaptosomes in this fraction [5].

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Initial attempts to measure [\(3^H\)]nicotine binding to Percoll gradient fractions resulted in the recovery of more binding sites than were present in the S1 fraction applied to the gradient. This paradox was explained by the observation that [\(3^H\)]nicotine exhibits specific binding to Percoll itself. This binding was displaced by nicotinic ligands (nicotine, carbamylcholine, dimethylphenylpiperazinium) but not by dopamine or atropine. [\(3^H\)]Methylcarbamylcholine, an alternative high-affinity nicotinic agonist for labelling brain nAChR, also showed specific binding to Percoll. This tendency presumably reflects binding to the silica core of the Percoll particles, as [\(3^H\)]nicotine is well known to bind specifically to glass [6]. Experiments were then carried out in which Percoll (final concentration 23%, v/v) was added to rat brain S1 fraction, and the preparation was assayed for