Mini Review

Biochemical events associated with rapid cellular damage during the oxygen- and calcium-paradoxes of the mammalian heart

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Summary. The O_2- and Ca^{2+}-paradoxes have a number of features in common and it is suggested that release of cytosolic proteins in both paradoxes is initiated by the activation of a sarcolemma NAD(P)H dehydrogenase which can generate a transmembrane flow of H^+ and e^- and also oxygen radicals or redox cycling which damage ion channels and membrane proteins (phase I). Entry of Ca^{2+} through the damaged ion channels then exacerbates the damage by further activating this system, either directly or indirectly, and the redox cycling and/or oxygen radicals cause further damage to integral and cytoskeletal proteins of the sarcolemma resulting in microdamage to the integrity of the membrane (phase II) and the consequent release or exocytosis of cytoplasmic proteins and, under specialised conditions, the blebbing of the sarcolemma. The system may be primed either by removal of extracellular Ca^{2+} or by raising $[Ca^{2+}]_i$ by a variety of measures, these two actions being synergistic. The system is initially activated in the Ca^{2+}-paradox by the membrane perturbation associated with removal of extracellular Ca^{2+}; prolonged anoxia in the metabolically active cardiac muscle causes a depletion of the ATP supply, particularly in the absence of glucose, and hence a rise in $[Ca^{2+}]_i$ in phase I of the oxygen paradox with the consequent activation of the NAD(P)H oxidase at the sarcolemma. Oxygen radicals are probably generated in both paradoxes and may have a partial role in the genesis of damage, but are not essential in the Ca^{2+}-paradox which continues under anoxia. Massive entry of Ca^{2+} also activates an intracellularly localised dehydrogenase (probably at the SR) which produces myofilament damage by redox cycling.

Key words. Cardiac muscle; cell damage; calcium; calcium-paradox; oxygen-paradox; oxygen radicals.

Independent damage pathways in muscle cells

The rapid efflux of cytosolic proteins (usually measured as the release of creatine kinase, CK) is a characteristic feature of the damaged mammalian heart, following both ischaemic and experimental interventions. In spite of intensive study, the biochemical events underlying this damage to the sarcolemma remain unclear.

Experimental evidence suggests that rapid cellular damage (with a timecourse of minutes) in mammalian skeletal and cardiac muscles have many features in common: (i) characteristic patterns of ultrastructural damage which may involve both the degradation of relaxed myofilaments and also hypercontraction with blurred Z-lines, these two types of damage frequently occurring in adjacent sarcomeres, (ii) massive release of CK, (iii) changes in the concentration of free calcium ($[Ca^{2+}]_i$) or in Ca^{2+}-fluxes can initiate rapid cellular damage.

These two pathways of muscle damage, namely sarcolemma breakdown and the destruction of the myofilament apparatus, are apparently spatially separated and largely independent in both skeletal and cardiac muscles: (i) Chlorpromazine and nordihydroguaiaretic acid (NDGA) inhibit CK release whilst myofilament damage is unaffected when $[Ca^{2+}]_i$ is raised in skeletal muscle [1], (ii) treatment of patients with Duchenne muscular dystrophy or polymyositis with steroids reduces plasma CK activity without affecting the course of the disease [2, 3], (iii) exposure of mouse soleus muscle in vitro to a variety of experimental protocols with anoxia and excessive contractile activity shows that extensive myofilament damage either precedes CK release or that there is no accompanying release of CK [4], (iv) perfusion of the isolated rat heart with menadione or phenazine methosulphate (PMS) causes severe ultrastructural damage and impairment of contractile function, but no release of CK [5].

Since inhibitors of phospholipase A_2 (PLA_2) and of the enzymic oxidation of arachidonic acid (AA) via the lipoxygenase pathway protect against sarcolemma breakdown in mouse soleus muscle in vitro [1], it is possible that the pathway that culminates in CK release in skeletal muscle is: Ca^{2+}-activation of PLA_2, activation of lipoxygenase, the generation of oxygen metabolites and redox cycling which lead to the molecular damage of membrane proteins and to the efflux of CK. However, the inhibitors used may have other effects and there is certainly no corresponding experimental evidence that lipoxygenase activity has a major role in initiating events that lead to CK release in mammalian cardiac muscle [5].

The O_2-paradox and the Ca^{2+}-paradox of the mammalian heart

The O_2- and Ca^{2+}-paradoxes are two familiar models for the study of CK release in the perfused mammalian heart. Each has two phases. In phase I of the O_2-paradox the heart is made anoxic or ischaemic for 30–40 min and no damage is detectable; however CK release rapidly ensues when O_2 is returned (phase II). In phase I of the

Ca^{2+}-paradox, the heart is perfused with Ca^{2+}-free saline for 2–10 min and no damage is detectable; CK release occurs within 90 s when Ca^{2+} is returned in phase II[6]. The system is primed in phase I of both paradoxes, but sarcolemma damage does not normally occur until phase II. There are no satisfactory inhibitors for either paradox and a current controversy concerns whether these two different paradoxes are facets of the same problem or whether there are two different underlying biochemical pathways which have the following features:

(i) Substantially reduced release of CK was observed in phase II of the Ca^{2+}-paradox[7,8] if the cells were apparently depleted of high-energy phosphates.
(ii) Inclusion of 11 mM glucose in the perfusion medium provided protection against the O_2-paradox in some rodent species, but not in others[9].
(iii) Incubation of cardiac myocytes at pH 6.2 to 6.8 is protective against the O_2-paradox[10].
(iv) Whereas no CK release occurs during phase I of the Ca^{2+}-paradox of rat heart, rabbit and guinea pig hearts release CK from the onset of exposure to a Ca^{2+}-free solution[11].

The effect of temperature on the paradoxes

The close similarity in the characteristic effects of temperature on the two paradoxes may provide a key to understanding the sequence of biochemical events. If the heart is maintained at 33 °C or below during phase I of either paradox, no CK release occurs during phase II; CK release increases dramatically when the temperature during phase I is increased from 33 to 37 °C[6]. However, the dependence of the Ca^{2+}-paradox upon temperatures above about 33 °C is not absolute; CK is released if the period of Ca^{2+}-free perfusion is increased to 20 min at 25 °C or to 40 min at 20 °C[12]. Phase II of both paradoxes is markedly less sensitive to the temperature of the reperfusion fluid; increasing the reperfusion temperature from 4–20 °C produces only a 1.5-fold rise in CK release and there is little further increase in release in the temperature range from 20° to 37 °C[6].

The effects of the Ca^{2+}-paradox are much more drastic than those of the O_2-paradox since the rate of CK efflux is nearly threefold and a very short period of Ca^{2+}-free perfusion is sufficient to produce damage. Nevertheless, the remarkably close similarity between the temperature sensitivities of the two paradoxes strongly suggests a correspondence between their biochemical pathways, and the limited effects of temperature at 20–37 °C in phase II indicates that a common, specialised mechanism underlies the rapid sarcolemma breakdown in both paradoxes. The profiles for the temperature-dependent transitions in phase I for the O_2- and Ca^{2+}-paradoxes are essentially superimposable[6] suggesting that only events during this priming phase are dependent upon the mobility of key molecules in the sarcolemma.

Phase I of the O_2-paradox

Oxygen radicals have been detected in the O_2-paradox[13] and may be implicated in cellular damage[14]. Oxygen radical scavengers also provide partial, but not complete protection for the ischaemic heart in vitro[15–24] suggesting that damaging active oxygen metabolites are generated when O_2 is returned following activation of the biochemical system during phase I. The protective effect of a lowered pH_o[10] may be related to an inhibitory effect on superoxide (O_2^-) production. Perfusion of the heart with enzymatically generated oxygen radicals, of which H_2O_2 is the most important component, causes a rise in $[Ca^{2+}]_i$ and ultrastructural damage[16,25,26]; such findings suggest that extracellularly generated H_2O_2 can damage cation-channels or cation pumps in the sarcolemma, thereby causing depolarization or a rise in $[Ca^{2+}]_i$. The most probable identity of a system that can potentially generate oxygen radicals at the sarcolemma is a vectorially-organised, transmembrane NAD(P)H dehydrogenase localised at the sarcolemma which is linked to electron and H^+ transport[27] and which is probably associated with the pentose phosphate pathway[28]. An NADPH dehydrogenase with these general properties is present in phagocytic cells where it can be induced to generate superoxides[27], and in thyroid where it generates H_2O_2 and requires Ca^{2+} for activity[29].

Regulation of NAD(P)H dehydrogenases and the generation of oxygen radicals

Although the properties of NAD(P)H dehydrogenases in most cells are not fully understood, there is accumulating evidence concerning the mechanisms by which the NADPH oxidase of phagocytes is activated and regulated. It is believed[30] that Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C) plays an essential role because: (i) protein kinase C activators stimulate O_2^- generation[31]; (ii) NADPH oxidase is phosphorylated by purified protein kinase C[32] and when the phagocytes are stimulated in vitro[33]; (iii) NADPH oxidase is activated by purified protein kinase C[34]. It is concluded that there are at least two types of association of protein kinase C with the cell membrane in phagocytes; one type is a reversible, Ca^{2+}-induced membrane binding that can be dissociated by chelators and the other is a phorbol 12-myristate 13-acetate-induced (stabilized) membrane binding that is stable to chelators and high salt concentrations, but can be dissociated with detergents[30].

Receptor-induced activation of protein kinase C in phagocytes is proposed to involve phosphatidylinositol hydrolysis resulting in an increased diacylglycerol formation and, thereby, protein kinase C activation, and inositol trisphosphate formation inducing an elevation of intracellular Ca^{2+}. The system is mimicked in vitro by TPA activation of protein kinase C and by elevation of $[Ca^{2+}]_i$ with A23187[30]. O_2^- production by NADPH

oxidase of membrane-rich fractions of phagocytic cells is also enhanced by Ca^{2+} (dissociation constant = 1.9×10^{-6} M)[35].

Is protein kinase C activity implicated in myocardial cell damage? Activators of protein kinase C enhance CK release from hypoxic cultured mouse myocardial cells as well as inducing a rise in pH_i and $[Ca^{2+}]_i$[36], and it is noteworthy that heart protein kinase C activity increases in cardiomyopathic hamsters during the progression of the disease, rising twofold by mid-stage[37].

Activation of the NADPH oxidase of phagocytic cells is dependent on membrane fluidity and is modified by membrane perturbation. The anionic detergent sodium dodecyl sulphate (SDS) elicits NADPH-dependent O_2^- production in a cell-free system derived from macrophages[38]; AA and other unsaturated fatty acids also activate the oxidase and this activation is strongly temperature-dependent[39]. It is proposed that both SDS and fatty acids act by stimulating a common mechanism that is dependent on a membrane-associated component and a soluble cytosolic factor. The former represents a membrane-associated component in an amphiphile-activated electron transport chain from NADPH to O_2[40]. Protein kinase C inhibitors cause a marked inhibition of O_2^- generation in neutrophils (thereby confirming a role for protein kinase C in the signal transduction process) and this is a highly temperature-sensitive event. No significant inhibition is observed if the temperature is lowered from 37 °C to only 35 °C[41] and this marked temperature-sensitivity corresponds with that of phase I of the O_2- and Ca^{2+}-paradoxes.

The NADPH oxidase of phagocytic cells is also stimulated by hypotonic media, and activity becomes normal again when the cells are returned to isotonic conditions; the activation is believed to be associated with a conformational change of the plasma membrane[42]. Correspondingly, hyperosmotic solutions (with added dextran, mannitol or polyethylene glycol) provide significant and substantial protection for the reoxygenated anoxic rat heart[43].

There are at least two ways in which such a NAD(P)H oxidase/protein kinase C complex could be primed during phase I of the O_2-paradox:

(i) Glycolysis will continue under anoxia, but the oxidative branch of the pentose phosphate pathway will be inhibited. The return of O_2 to the heart (phase II) may serve as the stimulus for the reactivation of the pentose phosphate pathway and hence the activation of the NAD(P)H dehydrogenase and the associated transmembrane electron flow.

(ii) More importantly, oxidative phosphorylation will be blocked under anoxia or ischaemia and ATP levels will fall under prolonged perfusion (40 min), so that Ca^{2+}-efflux and Ca^{2+}-uptake will be slowly impaired, and a substantial increase in free $[Ca^{2+}]_i$ has been measured in hearts under total ischaemia[44]. The protective effect of 11 mM glucose in some species[9] is explicable as the maintenance of the glycolytic supply of ATP and of $[Ca^{2+}]_i$ homeostasis. A local rise in $[Ca^{2+}]_i$ under anoxia probably activates or primes an NAD(P)H oxidase/protein kinase C complex and the return of O_2 allows the production of O_2^- (fig.).

Phase II of the O_2-paradox

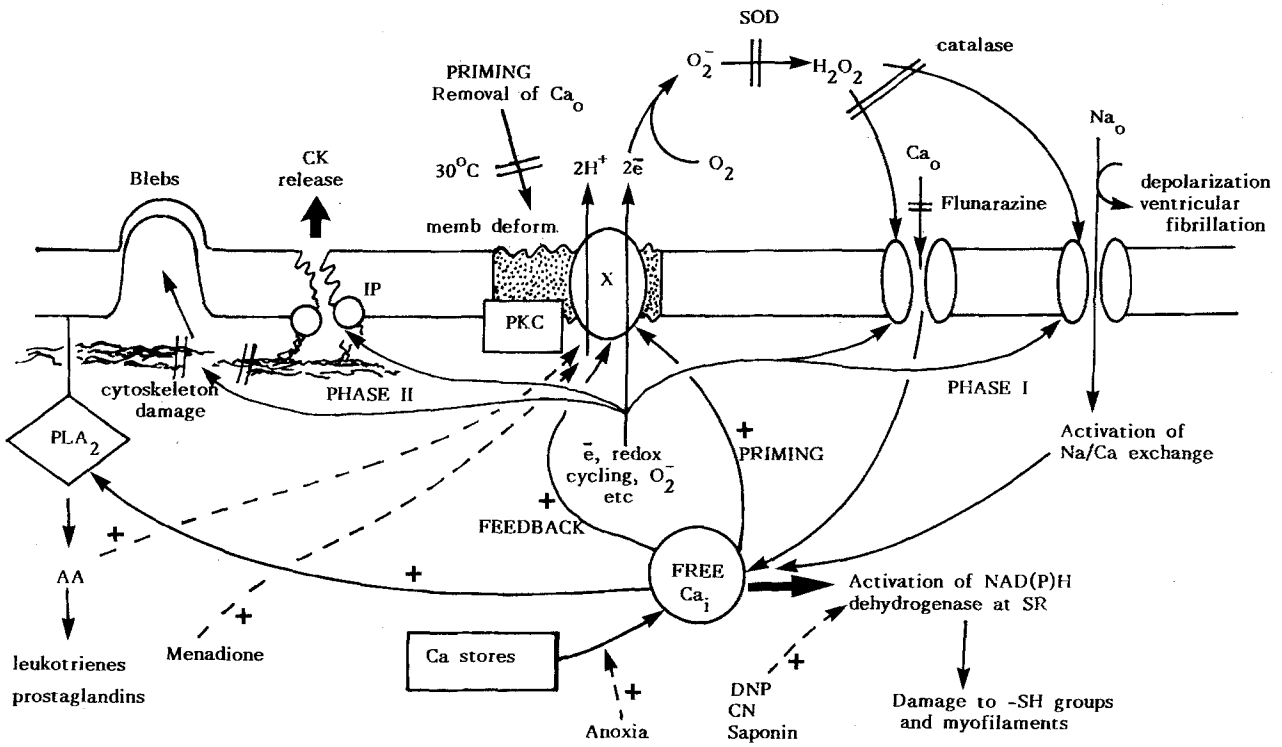
When O_2 is returned, the transmembrane flow of electrons produced by a NAD(P)H dehydrogenase can generate O_2^- and other O_2 metabolites at the sarcolemma and these can produce molecular damage at short range in adjacent proteins[45], such as ion channels and their regulatory proteins, cation pumps[46,47] and the subsarcolemma cytoskeletal network (fig.).

The Ca^{2+} slow channel is a complex structure, perhaps consisting of several proteins including two regulatory proteins, one stimulatory and one inhibitory, both of which may require phosphorylation in order to express their regulatory function[48]. Thus, G proteins regulate cardiac Ca^{2+}-channels directly as well as indirectly via activation of cytoplasmic kinases[49]. Hence a number of potential targets exist for the damaging action of O_2 metabolites, all of which may result in the modified cation permeability of the muscle cells. Changes in monovalent cation permeability will contribute to the characteristic reperfusion-induced ventricular fibrillation[14] (fig.).

Extracellular superoxide dismutase and catalase provide partial protection against reperfusion injury[15,16,21–24], suggesting either that the transmembrane movement of electrons generates extracellular O_2^- or that O_2^- generated intracellularly passes out via anion channels; in either case, the molecular damage would be produced at the extracellular entries of the cation channels (fig.). These events are modelled in experiments where the extracellular generation of H_2O_2 at high concentration causes cellular damage in perfused hearts that is protected by catalase[16,25,26]. Equally, since oxygen radicals artificially-generated intracellularly can damage the subsarcolemma cytoskeleton (see below) it is evident that electron flux and redox cycling can also produce damaging effects entirely within the cell membrane.

It is possible that damage to structural proteins associated with the sarcolemma and to the subsarcolemma cytoskeleton is sufficient to cause release of CK in certain circumstances (see below), the return of molecular oxygen being all that is required to generate sufficient damaging oxygen radicals. Recent studies show that in the reperfused rat heart after regional ischaemia, free radicals and the influx of Ca^{2+} act as simultaneous and interacting triggers for CK release[14,36]. Furthermore, artificially-generated O_2 radicals in isolated cardiomyocytes cause contraction and cellular damage via damaged Ca^{2+}-channels[50].

Events during phase II are rapid and complex but entry of Na^+ and Ca^{2+} is a feature of both O_2- and Ca^{2+}-



Suggested pathways of cellular damage in cardiac muscle cells associated with the activation of a NAD(P)H dehydrogenase (X) vectorially mounted in the sarcolemma which is capable of generating electrons and redox cycling. The system can be primed by removal of extracellular Ca or by raising $[Ca]_i$ during phase I when ion channels and pumps at close range are damaged by the electrons generated. Priming is markedly temperature-sensitive. The system is further activated by the consequent elevation in $[Ca]_i$ (phase II) when integral proteins of the sarcolemma and the cytoskeleton are damaged, resulting in microlesions and release of cytosolic proteins. Activation of X by menadione causes damage to the subsarcolemma cytoskeleton and membrane blebs. PKC = protein kinase C.

paradoxes, presumably via damaged cation channels or via impaired cation pumps and exchange mechanisms. It is suggested that it is this change in $[Ca^{2+}]_i$ (which has been measured by Ikeda et al.[36]) that is the major trigger for the pathways leading both to myofilament damage and to CK release (fig.). The latter process is relatively temperature-insensitive and events in phase II are dramatically fast.

Ca^{2+} could have positive feedback action (phase II) in initiating or augmenting sarcolemma breakdown in the following ways:

(i) Further, direct activation of the NAD(P)H oxidase/protein kinase C complex.

(ii) The sarcolemma Ca^{2+}-pump could be linked to and driven by the NAD(P)H oxidase[27] which will be further stimulated as the Ca^{2+}-pump attempts to correct the elevated $[Ca^{2+}]_i$.

(iii) Activation of a Ca^{2+}-dependent PLA_2, so producing AA which can directly stimulate the release of O_2^- from phagocytic cells via a PLA_2 pathway[51] by converting, with the aid of a cytosolic factor, the NADPH oxidase from an inactive to active form[52]; ATP is not required and protein kinase C is not directly involved[39]. Mepacrine, a PLA_2 inhibitor, reduces O_2^- production in neutrophils[53] but does not prevent damage in the Ca^{2+}-paradox of rat heart (Daniels and Duncan, unpublished).

(iv) Arachidonic acid acts as the substrate for the lipoxygenase pathway which may be responsible for the release of CK in mammalian skeletal muscle, since the lipoxygenase inhibitor NDGA provides almost complete protection[1]. However, there is currently no evidence that active metabolites of lipoxygenase activity are implicated in rapid cellular damage in cardiac muscle[5].

Thus, although leukotrienes and prostaglandins are produced in damaged muscles, suggesting that PLA_2 and the lipoxygenase and cycloxygenase pathways are activated, and although AA acts as an intracellular activator of O_2^- generation in macrophages and as a modulator of the cardiac K^+ channel, it is probable that Ca^{2+}-activation of PLA_2 has only a secondary feedback role in the production of severe damage. It may be of greater importance in the milder events of the O_2-paradox.

The mechanism by which cytosolic proteins are released at the sarcolemma

Although release of cytosolic proteins is a feature of damage in both skeletal and cardiac muscles, the under-

lying biochemical mechanism remains unknown. The primary targets for attack at the sarcolemma are either the phospholipid domain via lipid peroxidation (for which there is no convincing evidence[5, 54]) or the sarcolemma proteins. Studies with the action of PMS and anthracyclines suggest that oxygen metabolites and redox cycling can effect damage to the subsarcolemma cytoskeleton and to sarcolemma proteins. Thus, blebs and protrusions of the sarcolemma have been found in cells undergoing severe damage such as cardiac myocytes[55, 56], isolated skeletal muscle cells (Byrne and Duncan, unpublished) and hepatocytes[57, 58]. Surface blebbing is an early consequence of hypoxic injury in cells[59–61] and a rise in cytosolic free [Ca] has been suggested as the stimulus for bleb formation in certain circumstances[59–61]. Substances that modify cytoskeletal proteins cause similar cell surface alterations in hepatocytes[62], suggesting that the formation of blebs is related to a modification of the cytoskeleton.

PMS stimulates the NAD(P)H oxidases of the plasma membrane, thereby generating O_2^- intracellularly[27, 28] and causing cytotoxicity and a loss of sulphydryl groups in hepatocytes[51]. It also causes ultrastructural damage and surface blebs which are frequently associated with underlying hypercontraction bands in diaphragm[63] and amphibian[64] skeletal muscle, as well as in the perfused amphibian heart[65]. However, in the Langendorff-perfused rat heart[5] and mouse soleus muscle in vitro (McCall and Duncan, unpublished) PMS causes myofilament damage but neither CK release nor the formation of surface blebs occurs.

Menadione and other quinones can undergo one-electron reduction to form a semiquinone radical, the process being catalysed by a variety of flavoenzymes, including NADPH-cytochrome P-450 reductase; they can also undergo a two-electron reduction catalysed by NAD(P)H: (quinone acceptor) oxidoreductase[66]. In hepatocytes, menadione causes by oxidative processes rapid changes in intracellular thiols and Ca^{2+}-homeostasis which are associated with changes in the cytoskeleton by actin cross-linking and the consequent formation of blebs[67]. Menadione also causes severe ultrastructural damage in mouse diaphragm and soleus muscle in vitro and in the perfused rat heart. The damage is accompanied by large and characteristic surface protrusions and blebs in diaphragm[68] and rat heart[5] but not in soleus muscle, whereas menadione causes CK release in soleus muscle (McCall and Duncan, unpublished) but not in rat heart[5]. These actions of menadione on skeletal muscle are independent of extracellular Ca.

Such findings suggest that the artificial production of oxygen metabolites and redox cycling by PMS or menadione can cause release of CK and also damage to the proteins of the cytoskeleton which results in the formation of membrane blebs, particularly when accompanied by the contraction of the cell. Both phenomena appear to be two facets of the same underlying process; bleb formation occurs, at least initially, with an intact sarcolemma, whereas the release of cytosolic proteins may be dependent on micro-scale lesions of the sarcolemma that are consequent on damage to its integral proteins (fig.).

Hepatocytes rapidly develop blebs after the addition of CN^- and iodoacetate but $[Ca^{2+}]_i$ remains unchanged[58] and this effect may correspond with the reported direct activation of the NADPH oxidase of phagocytes by CN^- or dinitrophenol[69]. Neutrophils contain a compartment, formed of secretory granules, that is completely exocytosed when O_2^- production is stimulated. The secretory granules are localized close to the plasma membrane[70] and these findings have similarities with the vacuoles found beneath the sarcolemma of damaged cardiac muscle cells[6]. Perhaps cytosolic proteins are released from damaged muscle via an abnormal form of exocytosis; a suggestion that would explain why only a small proportion of the total CK content is lost during muscle damage.

Phase I of the Ca^{2+}-paradox

In spite of many fundamental similarities, the Ca^{2+}-paradox has some clear differences from the O_2-paradox: removal of extracellular Ca^{2+} for only 30 s[71] is adequate to prime the system (in contrast with 40 min anoxia) and the damage in phase II is markedly more severe. For a 2-min exposure $[Ca^{2+}]_o$ needs to be below about 8×10^{-7} M[5]; for 10-min exposure $[Ca^{2+}]_o$ can be as high as 5×10^{-5} M. The sarcolemma is protected if Sr^{2+} is substituted for extracellular Ca^{2+}, but the inclusion of ATP (10^{-6} M to 10^{-4} M) in the perfusion medium greatly exacerbates the release of cytosolic proteins[71].

No overt ultrastructural changes are detectable at the sarcolemma during the first three min of Ca^{2+}-free perfusion, but prolonged perfusion causes a number of changes, including the separation of the external lamina from the surface coat of the sarcolemma which results in the formation of fluid-filled 'blebs'[72] that are different from those found following exposure to menadione or PMS but are similar to the vacuoles[6] described above. It is suggested that removal of extracellular divalent cations produces membrane deformation that leads to the molecular perturbation and activation of a sarcolemma NAD(P)H dehydrogenase during the first 2 min of Ca^{2+}-free perfusion, thereby causing damage to ion channels. Oxygen radicals artificially generated by photo-excitation of rose bengal induce ultrastructural damage in rat cardiomyocytes via Ca^{2+} entry through modified Ca^{2+}-channels[50].

Perfusion with superoxide dismutase, catalase and various scavengers and inhibitors of oxygen metabolites failed to protect significantly against even mild conditions of the Ca^{2+}-paradox in rat heart[5] and the Ca^{2+}-paradox occurs even under anoxic perfusion with glucose[73] so that, unlike the O_2-paradox (where the pro-

tection is only partial), molecular oxygen and oxygen radicals are not implicated in either phase I or phase II of the Ca^{2+}-paradox. Damage of the sarcolemma proteins must be achieved directly, perhaps by electron transfer or by redox cycling.

CK release in the rat heart perfused with menadione or with agents[74] believed to raise $[Ca^{2+}]_i$ is triggered or augmented by perfusion with anoxic or glucose-free media, both of which will raise $[Ca^{2+}]_i$ (Daniels and Duncan, unpublished). It is concluded, therefore, that the damage system can be primed either by removal of extracellular Ca^{2+} (membrane perturbation; phase I of the Ca^{2+}-paradox) or by raising $[Ca^{2+}]_i$.

Phase II of the Ca^{2+}-paradox

Na^+ enters during phase I[75] and there is no doubt that there is a massive entry of Ca^{2+} during phase II, and it has been suggested that $[Ca^{2+}]_i$ rises via Na^+/Ca^{2+} exchange[75]. This may have a contributory effect, but it seems unlikely that sufficient Na^+ could enter during a 30 s- or 2-min Ca^{2+}-free perfusion, except via damaged cation channels which would provide the major route for Ca^{2+}-entry. A number of studies show that verapamil and other Ca^{2+}-antagonists can have modifying effects on the Ca^{2+}-paradox, but do not prevent CK release, indicating that inhibition of Ca^{2+}-entry via undamaged slow channels does not protect[73]. Ca^{2+}-antagonists that are not active on the slow Ca^{2+}-channel (flunarizine) provide much better protection against the damage produced by artificially generated O_2 radicals than do genuine slow Ca^{2+}-channel blockers[50].

Massive Ca^{2+}-influx in phase II triggers both the severe damage to the myofilament apparatus and the dramatic release of CK; Sr^{2+} substitutes in the activation of both pathways[72]. Ca^{2+} or Sr^{2+} therefore again provide a positive feedback mechanism for greatly exacerbating sarcolemma damage and could act via the same excitatory mechanisms as suggested above for the O_2-paradox. CK release occurs during Phase I of the Ca^{2+}-paradox in rabbit and guinea pig hearts[11]; furthermore, Ca^{2+}-free perfusion alone after anoxia will directly trigger release of CK in rat hearts[76] (Daniels and Duncan, unpublished). Hence the events in phase I can be sufficient to cause sarcolemma damage without the feedback effects of Ca^{2+} entry in phase II.

Damage to the myofilament apparatus

Although the ultrastructural changes that occur in the cellular damage of cardiac muscle have been well documented[77], the underlying biochemical mechanisms are not well understood and most studies have concentrated on damage at the sarcolemma. Since the events of rapid, Ca^{2+}-activated damage and the patterns of myofilament ultrastructural degradation are very similar in skeletal and cardiac muscle[78] it is possible that there is a common underlying sequence of biochemical events. In chemically-skinned amphibian skeletal muscle, identical patterns of myofilament damage were directly and rapidly triggered by rises in $[Ca^{2+}]$ or $[Sr^{2+}]$, or by dinitrophenol or CN^- at zero $[Ca^{2+}]$, or by membrane perturbation, features that are shared by the NADPH oxidase of phagocytic cells. However, these effects continue under anoxia and in the presence of scavengers and inhibitors of oxygen radicals. Thiol oxidizing agents (N-ethyl maleimide, diamide) also produce characteristic ultrastructural damage at zero $[Ca^{2+}]$[79,80] and it is suggested that myofilament damage in skeletal muscle is initiated by redox cycling generated by a system located at the SR that preferentially attacks key -SH groups. Since menadione produces identical myofilament damage in cardiac[5] and skeletal[68] muscle it is further suggested that it also stimulates redox systems on the SR via an attack that is analogous to its action on NADH oxidases of the ER of liver.

O_2-paradox vs Ca^{2+}-paradox

The many points of similarity between the two paradoxes, namely the patterns of ultrastructural damage and release of cytosolic proteins, the common differential sensitivity to temperature of the two phases, the requirement for priming, the extreme rapidity of damage in phase II, the central role of intracellular Ca^{2+} and the sensitivity of the sarcolemma to fluidity and perturbation in phase I, provide circumstantial evidence that suggests that the same underlying biochemical mechanisms operate in both. And yet there are puzzling differences. O_2 radicals are apparently partly involved in the O_2-paradox since phase II is triggered by O_2, partial protection or amelioration is provided by O_2 radical scavengers, O_2 metabolites have been detected intracellularly during damage and the paradox can be partly simulated by extracellularly generated H_2O_2; conversely the Ca^{2+}-paradox (and the myofilament damage of skinned skeletal muscle) can proceed under anoxia and is unaffected by O_2 radical scavengers[5] and the transfer of electrons must proceed directly. However, oxygen radicals are generated during the Ca^{2+}-paradox under normal oxygenation, as shown by a rise in malondialdehyde production, but their inhibition does not prevent cellular damage[54]. Such findings support the concept of a common mechanism underlying phase I of both paradoxes and confirm the suggestion that oxygen metabolites may not have a major role. Neither paradox has an obligatory requirement for entry of extracellular Ca^{2+} since CK release is recorded in Ca^{2+}-free saline; a rise in $[Ca^{2+}]_i$ from intracellular storage sites is sufficient to initiate damage. There is no firm evidence concerning the biochemical identity of the damage system that can be primed either by the brief removal of extracellular Ca^{2+} or by raising $[Ca^{2+}]_i$ locally, the two effects being synergistic. Nevertheless, the observation that menadione causes identical damage and re-

quires similar priming by removal of extracellular Ca^{2+} provides additional circumstantial evidence that the biochemical systems that initiate the damage of both the sarcolemma proteins and the myofilament apparatus have features in common with the redox systems of the ER and with the O_2^--generating systems of phagocytic cells.

There is some evidence concerning the properties of this damage system. CN^- and some metabolic inhibitors apparently provide protection against the Ca^{2+}-takeover.[7,8,81,82]. Activators of protein kinase C induce a rise in pH_i in cultured mouse myocardial cells in the presence of A23187 by activating amiloride-sensitive Na^+/H^+ exchange. They also enhance CK release under hypoxia and this effect is also markedly suppressed by amiloride[36], and damage is substantially reduced in both the Ca^{2+}-paradox[8] and O_2^--paradox[82] when permeant anions capable of proton donation (HCO_3^-, $H_2PO_4^-$) are omitted from the perfusion saline. The NADPH oxidase of neutrophils is electrogenic and associated with an H^+ channel[83,84] and such studies also suggest a key role of such a system, regulated by protein kinase C and generating transmembrane electron and proton flow, in the genesis of cellular damage in cardiac cells.

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Research Articles

Vagal afferent innervation of the pylorus and the upper small intestine studied in the rat with the horseradish peroxidase technique

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Summary. The neuronal tracer horseradish peroxidase was injected into different segments of the gastrointestinal in the rat, in order to study the vagal afferent innervation. In the nodose ganglia the extent of labeling was greater in the experiments on the gastric antrum and pylorus than in the experiments on the first part of the small intestine. Vagal afferents are scarce in the upper duodenum and originate mainly from the left nodose ganglion.
Key words. Horseradish peroxidase; vagus nerve; nodose ganglion; stomach; pylorus; duodenum; rat.

Recent electrophysiological studies have clearly proved the existence of many kinds of receptors such as mechanoreceptors, chemoreceptors, thermoreceptors and osmoreceptors in the intestinal wall[1]. These receptors probably play important physiological roles. Vagal receptors are particularly abundant in the duodenum of the cat and are distributed in all layers[2-4]. In this animal, the use of the horseradish peroxidase (HRP) technique has shown that the first part of the small intestine receives many vagal afferent fibers; in fact, a few hundred labeled neurons have been found in the nodose ganglia after injections of this neuronal tracer[5]. As for the duodenum, Lundberg et al.[6] have found HRP-positive neurons both in the left and in the right nodose ganglia of cat and guinea pig after injections of the marker at multiple sites in the visceral wall. The occurrence of the HRP reaction product on one side was prevented by crushing the cervical vagal nerve on that side. Unfortunately, these authors did not report either how many neurons were labeled or the number of HRP-positive cells in the left and right nodose ganglia.

The present study was carried out in the rat, employing the HRP tracing technique to provide clearer information on the distribution of duodenal afferents in the nodose ganglia. The pylorus and gastric afferents were also investigated for purposes of comparison with the innervation of the first part of the small intestine. The rat was chosen as the experimental animal because, even if it has been extensively investigated for the afferent and efferent organization of the vagus nerve[8-14], no data are available concerning the vagal afferents in the first part of the small intestine.

Materials and methods

Experiments were performed on 21 male Wistar rats, 300–350 g in body weight, anesthetized with intramuscular injection of 50 mg/kg ketamin chlorhydrate. Following laparatomy various quantities of a 20% (W/v) solution of HRP in H_2O were injected, with the aid of a dissecting microscope, through a Hamilton microliter syringe. The amount of the tracer administered depended on the area to be injected. In each injection, two HRP aliquots of 1 µl each were delivered, withdrawing the needle into nearby sites, to reduce the number of injections and thus to minimize the leakage of the neuronal tracer. For the same reason the zone was covered with a pledget after the injection, to avoid backward spreading