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IN VITRO EFFECTS OF CALCIUM LOADING AND 2, 4-DINITROPHENOL ON SARCOLEMMAL INTEGRITY OF SKELETAL MUSCLE IN ALBINO MOUSE.

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ABSTRACT

Experiments were undertaken to investigate the effects of calcium loading together with addition of the metabolic poison, 2, 4- dinitrophenol on sarcolemmal permeabilisation of albino mice skeletal muscle. Incubation with a 2 x 10^{-5} M solution of 2, 4- dinitrophenol for 120 minutes exerted only a slight effect on sarcolemmal permeabilisation (As assayed by penetration of the fluorescent dye, Reactive Orange). Subsequent incubations at higher concentration of 2, 4- dinitrophenol (2 x 10^{-4} M) for 120 minutes led to permeabilisation of the sarcolemmal in 28.3% of the cells. Sarcolemmal permeabilisation was restricted to the peripherally situated cells, although the depth of penetration of dye increased higher in concentration of 2, 4- dinitrophenol. It is possible that this pattern of damage distribution induced by calcium loading resulting from treatment with 2, 4- dinitrophenol is related to the limited diffusion distance of the latter, as the central fibres were not affected by such treatment. Damage was considerably reduced when the experiment was conducted with Ca^{2+} - free saline. Removal of extra cellular calcium resulted in permeabilisation of ٨,١% of the cells, compared with 2.4%, cell damage in the control experiment (2 x 10^{-4} M 2, 4- dinitrophenol, 120 min) and 28.3% in the corresponding third experiment. This increase in level of sarcolemmal permeabilisation could be attributed to damage induced by the action of 2, 4- dinitrophenol alone, and that extra cellular calcium may play an important role in cell death. Another possibility is that intracellular calcium leaking from the mitochondria may lead to cell damage.

INTRODUCTION

Impaired control of intracellular free Ca 2+ concentration is a ubiquitous characteristic of muscle cells undergoing necrosis. Numerous cell processes e.g.

excretion-contraction coupling, cell metabolism regulation, endocytosis and exocytosis are mediated by changes in concentration of intracellular free Ca 2+. Consequently, its precise regulation is extremely important to the cell (Bannister & Puplicove, 1993).

Mobilization of Ca 2+ can induce skeletal muscle necroses both in vivo and in vitro (Howl & Puplicover, 1990). Necrosis itself may be defined as, "a number of alterations in the morphology of cells after they've died by mechanisms that may / may not have anything to do with their transformation into necrotic debris." This distinction is practicable in a situation such as cell death without necrosis after removal from the body and fixation of tissue sections (Farber, 1982).

Cell death is frequently encountered in human disease and it may be attributed to number of causes e.g. ischemia, chemicals, viruses, radiation, toxins (Farber, 1981). An increase in cytosolic free Ca^{2+} concentration has been implicated in a range of clinical myopathic concentration such as Duchenne muscular dystrophy (Howl & Puplicover, 1989; Farber, 1981, 1982). Many muscle diseases show common pathological features, even though their aetoiology is different. The excessive release of intracellular enzyme is observed and probably indicates an increase in a number of myopathies and probably indicates an increase in sarcolemmal permeability (Wrogeman & Pena, 1976).

Influx of calcium across the injured membrane and along steep concentration gradients changes potentially reversible alterations to become irreversible injury causing cell death. This is associated with defects in oxidative phoshorylation caused by increased calcium in the mitochondria. It is still somewhat uncertain as to whether Ca^{2+} enters the cell through normal channels or through disruptions in the surface membrane (Jones et al, 1984). The calcium influx forces mitochondria to maintain calcium homeostasis by sequestering excess Ca^{2+} in the mitochondria. This is an energy process which may occur in preference to ATP formation (Farber, 1981). The ensuing lack of energy instigates a vicious cycle of events. While calcium overload is accelerated, the mitochondria begin to leak calcium, thus increasing the concentration of free, cytosolic calcium. The resulting organelle calcification leads to impairment of function and damages the structure of proteins and phospholipids (Oberc & Engel, 1977). Ultra structural studies show a short period of hypercontraction in the muscle fibres preceding cell death (Wrogeman & Pena, 1976).

The actual mechanisms through which Ca 2+ evokes structural damage to the cell are still subject to much controversy. It seems that there are at least 2 pathways that

culminate in cellular damage in skeletal muscle (Duncan,1989; Jackson,1990 Howl &Puplicover,1989;Howl& Puplicover, 1990).

Numerous tissues, including skeletal muscle, contain proteolytic enzymes which are activated upon an increase in intracellular calcium (Ishiuram et al. 1981), and Ca2+ - activation of Phosholipases (Particularly phospholipase A and possibly phospholipases C) and lipid peroxidation probably underlie the permeabilisation of the cellular membrane associated with elevated levels of Ca^{2+} (Jackson, 1990; Bannister & Puplicover, 1993).

2, 4-DNP is an uncoupling agent which disrupts the tight coupling of electron transport and phosphrylation (Stryer, 1988). Addition of 2,4 DNP to cells stops ATP synthesis by mitochondria without blocking their uptake of oxygen, Electron transport proceeds at a fast rate , but ATP is not formed by the mitochondrial ATPase as the proton-motive force across the inner membrane is dissipated (Stryer, 1988). The elevation of Ca^{2+} levels instigate Ca^{2+} uptake by the sarcoplasmic reticulum , (Bannister & Puplicover, 1995) thus increasing for ATP and reducing the ATP/ADP ratio. In addition to this, the increase in intramitochonrial Ca^{2+} affects the acceleration of enzymes utilized in oxidative phosphorylation. The resulting depletion of energy levels induces a cascade of deleterious events leading to eventual cell death (McCormack & Denton, 1993).

2, 4- DNP alone induces extensive myofilament degradation, resembling that seen with ionospheres such as A 23287 which acts in the absence of extra cellular Ca^{2+} from the sarcoplasmic reticulum. 2, 4-DNP promotes lysosomal labialization in living cells, produced by mitochondrial uncoupling and the release of stored calcium. This increase in calcium promotes lysosomal breakdown and the hydrolases released produce rapid myofilament dissolution (Duncan et al, 1980).

Many metabolic poisons such as 2,4-DNP have entry of calcium as a final common step in the sequence leading to cell death (Jones & Round, 1990). Extensive work has been conducted by Bannister and Puplicover (1993, 1995) and Howl and Puplicover (1989, 1990) using the calcium channel agonist Bay k 8644 (Bay). This dihydropyridine binds specifically to L-type voltage-dependent Ca^{2+} channels, stabilizing the open state and thus increasing calcium flow through the channel. Treatment of partially depolarized diaphragms with Bay induces ultra structural damage and severe membrane permeabilisation. A significant feature of Bay K 8644-induced uptake of fluorescent dye is that the fibres situated centrally within the diaphragm are more susceptible to membrane damage than the peripheral layers. Bannister and Puplicover (1995) postulate that this selective damage involves Ca^{2+}- Induced hypoxia. Hypoxia probably induces further Ca^{2+} accumulation (supported by the fact that this effect is inhibited in Ca^{2+}- free medium) and

contributes in association with Bay k 8644 to the failure of calcium homeostasis in the cell. .The present work was conducted to study the effect of calcium loading and uncoupling agent 2, 4-Dinitrophenol on sarcolemmal integrity of albino mice skeletal muscle in vitro.

MATERIALS AND METHODS

Diaphragm muscles from adult white male mice (6 - 7 weeks) were used. The mice were maintained under standard laboratory conditions with free access to food and water. Animals were killed by cervical dislocation and the diaphragm removed into standard physiological saline, The diaphragm was then cut into two hemi diaphragms and each was pinned, under minimal tension, onto a piece of dental wax (this was centrally perforated to allow aeration of the muscle). The muscle was incubated at 37°c in a water bath. This incubation medium consisted of a 60 ml circulation volume of saline, 20 ml of which comprised the working volume whilst the remainder was contained in a reservoir. The saline was aerated eighth 95:5 carbogen and it was circulated by a pump to give a perfusion rate of 3ml/min. Muscle was incubated for 100 – 120 minutes.

Physiological saline and preparation of 2,4- DNP and Reactive orange:

The composition of the physiological saline was as follows:

NaCL, (118m mol/L) ; KCL, (5.9 mol/L) MgSo4, (102m mol/L) ; NaHco3, (25m mol/L) NaH2PO4, (1.25m mol/L); glucose, (11.1m mol/L); CaCL2, (1.8m mol/L). The saline was equilibrated with carbogen and the pH was adjusted to 7.4. The saline was made up the day before its usage and stored at 4°c to ensure that it was fresh.

All the experiments described involved staining with Reactive Orange (Sigma Co.).0.05g of the fluorescent stain was dissolved in the circulating saline. When required (experiments 2, 3 and 4) , 0.05 g 2,4-dinitrophenol (Sigma) was dissolved in saline and subsequently diluted to give final concentrations of 2 x 10 – 5 M and 2 x 10 – 4 M and 2 x 10 – 4 M respectively. In the final experiment conducted, calcium free saline was used in the incubation process. This was achieved through the exclusion of calcium from the bathing medium and the addition of 0.5m mol/L EGTA (0 Ca 2+ buffer) to the circulating volume.

HISTOLOGICAL TECHNIQUES

The hemi diaphragms were bathed continuously in the appropriate solution. Following incubation, the muscle was washed with saline to remove any excess stain. It was then fixed in 2.5% glutaraldehyde in 0.1 m phosphate buffer, for at least 1 hour at 4°c.

The muscle was then unpinned and cut into 2 or 3 smaller peaces under a dissecting microscope. The muscle was then dehydrated in a series of increasing alcohol concentrations. Samples were allowed to remain off 15 minutes in each concentration. Two changes of histoclear (Cell Path plc) were required at hourly intervals to remove any remaining alcohol.Two changes of paraffin wax, also for 1 hour each at 60° C, allowed the wax to fully penetrate the muscle. Each sample was then embedded in fresh wax to produce a block which was left to set overnight at room temperature. After trimming with a razor blade, each block was sectioned transversely into 7 micrometer slices using the Spencer 820 microtome. The wax ribbons obtained were laid on to water covered slides and left to dry overnight on a hotplate (at a temperature just exceeding that of the human body i.e. > 37 ° c).

The wax was cleared from the slides by placing them in histoclear for 2 minutes. Sections were mounted in hystomount (Hughes and Hughes Ltd.) and left on a hotplate for 2 hours. Observations were made using the zees 111 Rs fluorescence microscope and subsequently photographed on Kodak T. Max 400 film.

CALCULATIONS AND STATISTICS

The percentage of stained cells was calculated by choosing areas at random and counting the numbers of stained and unstained cells in the field of view. The number of cells counted in each field of view varied from 60 to 192. A percentage of stained cells were calculated for each experiment. The mean ± standard error of mean (SEM) percentage permeabilisation was also calculated. The Chi-square test was then used to compare levels of permeabilisation for each experiment.

RESULTS

After incubation in standard physiological saline (containing Reactive Orange) for 100min transverse sections of the muscle of control animals showed that the majority of the fibres had a densely compacted appearance. Only a few fibres had taken up the fluorescent yellow/orange dye (Table 1), although slight fibre separation was evident. However, most of the muscle cells appeared green in colour when viewed with the fluorescence microscope. This pattern was represented in all the sections viewed and reflected in the relatively low % staining value of 2.4% (Table 1).

In diaphragms which were incubated for 120 min in saline containing 2×10^{-5} M 2,4-DNP (Fig, 1), 5.6 % staining of muscle fibres was seen. Although this is not a particularly high proportion of fibres, fibre separation had clearly been exacerbated by addition of the uncoupling agent. The stained fibres are peripherally situated in the muscle sections.

As the proportion of stained fibres in the latter experiment was not as high as perhaps expected, the concentration of 2, 4-DNP was increased to 2 $\times 10^{-4}$ M. This produced a significant increase in the proportion of fibres which had taken up the dye (28.3% - Table 1). Again, the stained fibres were restricted to the superficial layer. A high degree of fibre separation was evident (Fig, 2) as was the detachment of the sarcolemmal membrane .There appeared to be two distinct areas of muscle damage in this experiment, some of the sections were stained completely and showed severe fibre separation whilst the muscle cells in other sections had not taken up the dye at all and fibre separation was minimal. The distribution of stained cells did not appear to be random, and it's possible that these areas of myofibrillary clumping could be due to the action of nitric oxide and other possible mechanisms. It certainly seems that these areas of localized damage signify actual damage, and not mechanical damage resulting from the dissection.

To investigate whether calcium loading is indeed contributing to sarcolemmal permeabilisation and eventual cell death, an experiment was conducted in which hemi diaphragms were incubated in physiological saline containing 0 Ca^{2+} - EGTA and 2 X 10 $^{-4}$ M DNP Transverse sections of the muscle showed extreme fibre separation (Fig. 3) and 8.1% of the fibres had taken up the dye (Table 1) Once again, the fibres affected occupied a primarily peripheral situation.

Chi-square test showed that the values calculated for each experiment proved to be significantly different (< 0.001) from the control, especially when the muscle was incubated with 2 x 10^{-4} M 2, 4-DNP for 120 min (Table 1).

The Chi-square test was also used to compare the experiments to one another. The values calculated were significantly different ($p < 0.001$) when the third experiment was compared to the second and fourth experiments. Only when the second and fourth experiments were tested against one another a non significant value obtained ($P > 0.3 < 0.5$).

Table 1. Effect of 2,4 - Dinitrophenol and calcium loading on membrane permeability as assayed by uptake of Reactive Orange.

Incubation (time)	Staining (% cells)	Sections Cells	Chi- Square Value
Control (100 min)	2.4 ± 0.9	10/ 1000	-
2 x 10^{-5} mol/l 2, 4- DNP (120 min)	5.6 ± 1.1	10/ 1024	49*
2 x 10^{-4} mol/l 2, 4- DNP (120 min)	28.3 ± 9.7	10/ 1035	135*
2 x 10^{-4} mol/l 2, 4- DNP(120 min, 0 Ca^{2+})	8.1 ± 1.1	10/ 1078	32*

* Significantly different (at $P < 0.001$)

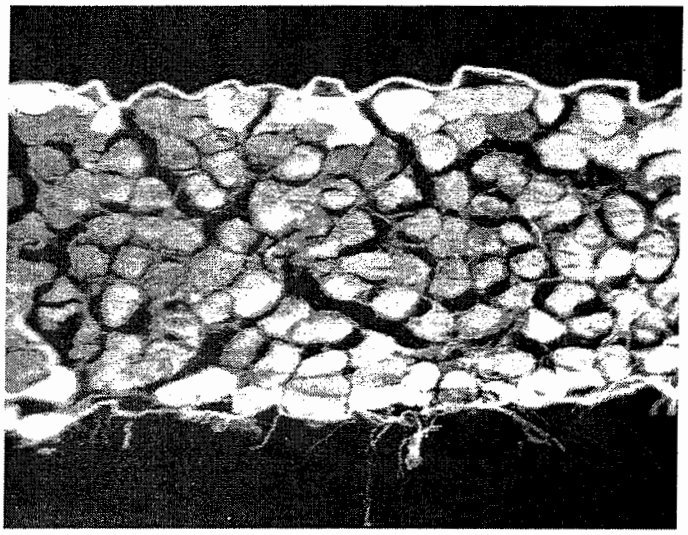

Fig. 1 . Saline + 2 x 10^{-5} m mol/L 2,4 - dinitrophenol for 12min. Fiber separation has occurred and the fluorescent dye has penetrated a few of the peripherally located fibers , X475.

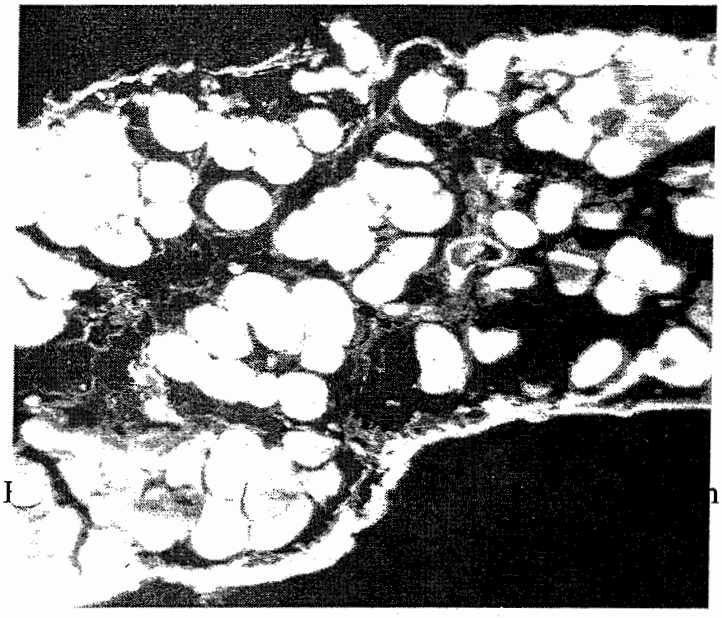

Fig. 2 . shows a very damaged area of muscle. All the fibers have taken up the dye and the fiber separation is extremely advanced, X475.

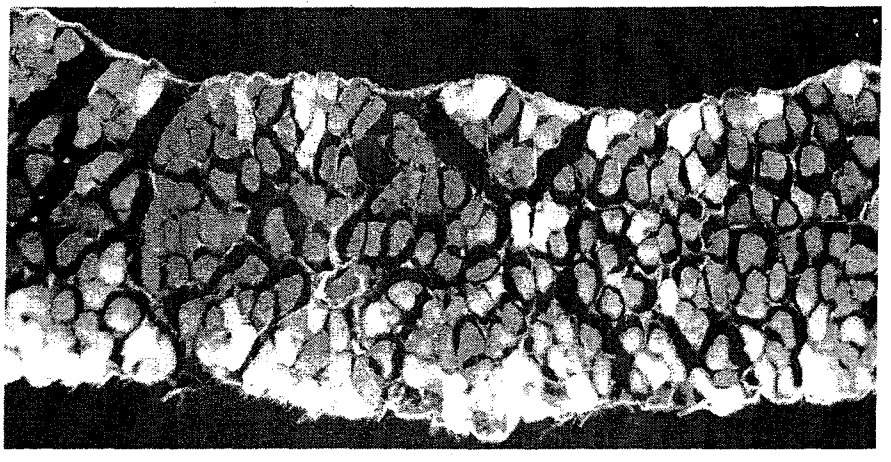

Fig. 3. Saline + 2x10 $^{-4}$m mol/L 2,4 dinitrophenol + 0 Ca^{2+} EGTA for 120min. Note the fiber separation and the fibres fluorescing primarily in the superficial layer of the muscle. These processes are in evidence even in the absence of extra cellular Ca^{2+}, X 475

DISCUSSION

The present results illustrates that treatment of mouse diaphragm in vitro with 2, 4-Dinitrophenol for 120 min results in a structural damage and permeabilisation of the sarcolemma.

Increasing the concentration of the 2, 4- Dinitrophenol from 2x 10 -5 mol/l to 2x 10 -4 mol/l produced a concurrent increase in the observed levels of damage. This is probably due to the elevated level of intracellular calcium taken up by the sarcoplasmic reticulum, and thus increasing demand for ATP and decreasing the ATP/ADP ratio (McCormack & Dentonm 1993). 2, 4-Dinitrophenol stops ATP synthesis by mitochondria and the resulting depletion of energy levels induces a cascade of deleterious events (Duncan, 1987).

In the control experiment 2.4% of fibres allowed entry of the Fluorescent dye, compared to 28.3 % staining when the hemi diaphragms were incubated with 2 x 10 -4 mol/l DNP, adjacent areas of clumped myofibrils were seem to be both stained and unstained. Bradley and Fulthorpe (1978), attributed this to condensation of myofibrils or some physiochemical change which increases the concentration of basic groups in the area. Bradley also suggests that the elevationof intracellular Ca 2+ in myofibrils acts as a mordant for the fluorescent dye. If this proved true, it would not be disadvantageous with regards to experimental accuracy since it tends to localize dye in the region of sarcolemmal damage through which it entered the muscle fibres. Histological examination of muscle

revealed that damage was concentrated in peripheral cell layers, and that depth of penetrated of the Reactive Orange dye increased with increase in the concentration of 2, 4-DNP (from 2x 10 -5 mol/l to 2x 10 -4 mol/l), indicating an increase in sarcolemmal permeabilisation, This pattern of distribution was consistent throughout all the experiments conducted.

Evidently, this pattern of damage occurred herein is markedly different to that seen in experiments using the calcium channel agonist, Bay k 8644 (Bannister & Puplicover, 1993 Howl & Puplicover, 1989, 1990).

With the latter agent, the fibres situated centrally within the diaphragm were more susceptible to membrane damage than the peripheral layers (Bannister & Puplicover, 1995). Whereas we observed that it was primarily the peripheral layers that were subject to staining. Fibre typing has provided no evidence that peripheral fibres differ in character from those deeper in the diaphragm (Howl & Puplicover, 1990).

This difference may be explained by the involvement of Ca 2+-induced hypoxia (mimicked by non - aeration of the bathing medium) in the experiments using bay k 8644. Hypoxia probably results in accumulation of Ca 2+ and works in parallel with bay k 8644 in the failure of calcium homeostasis (Bannister & Puplicover, 1995).

Incubation of hemi diaphragms in Ca 2+-free medium ameliorates damage, inferring that influx Ca 2+ may be significant in triggering permeabilisation, as shown by Jones and co-workers (1984). The level of permeabilisation was significantly different ($P< 0.001$) from that seen in the previous experiment. This finding may reflect that 2, 4-DNP alone is capable of damaging muscle cells (Duncan et al. 1980). It is also likely that intracellular calcium leaking from the mitochondria is responsible for some of the damage exhibited.

Later work by Duncan a Jackson (1987) reported that cellular damage in the presence of 2; 4-DNP was not decreased in the absence of extra cellular calcium. Evidently, this is an area subject to some conjecture. This apparent "Calcium Paradox" has been studied in some detail by Soza and co-workers (1986). They discovered that lowering the extra cellular calcium concentration appeared to deplete calcium from skeletal muscle sarcolemma and alter ion conduction. Reperfusion with Ca 2+ induced necrosis and hyper contraction of the muscle.

A number of theories are extant with regards to mechanisms involving muscle cell damage. Skeletal muscle contains cytoplasmic proteolytic enzymes that may be activated upon elevation of intracellular Ca 2+ levels (Ishiure et al. 1981), and activation of phospholipases and lipid per oxidation may lead to permeabilisation of cellular membranes associated with increased calcium levels inside the cell (Duncan, 1989; Duncan & Jackson, 1987, Jackson, 1990).

Calcium-induced membrane phospholipids hydrolyses via activation of phospholipase enzymes appears to play a key step in the process leading to loss of cell

viability and efflux of cytosolic enzymes, (Jackson et al. 1984; Jackson et al. 1987) although Ca 2+ -induced mitochondrial damage also occurs (Wrogemann & Pena, 1976). The activated phospholipase liberates fatty acids from membrane- bound triglycerides, which may directly affect the integrity of the cell membrane (Jones & Round, 1990). Free fatty acids have a detergent action, causing further damage to the membrane of the cell wall and the intracellular organelles.

Calcium accumulation during damage induces release of arachidonic acid, in particular from membrane phospholipids by activation of phospholipase A 4 (although phospholipase may be involved), (Jackson, 1990). The released arachidonic acid acts as a substrate for production of prostaglandin and production of highly active metabolites (e.g. leukotrienes) via Lipoxygenase activity.

So, it seems likely that production of phospholipase A 2 activity is responsible for cell death following intracellular Ca 2+ overload. However, modification of skeletal muscle fatty acid composition in order to alter production of phospholipase A2 activation makes the cell more susceptible to calcium overload, not less susceptible as perhaps expected. This may be because lipoxygenase enzymes act on a diverse range of unsaturated fatty acids, producing damaging metabolites. Alternatively, a process such as non-enzymic lipid peroxidation may be activated during the damaging process (Jackson, 1990). Lipid peroxidation and formation of OH- depend on the presence of transition metals (Halliwell and Gutteridge, 1984). It appears that is not the primary action of phospholipase A2 that induces damage, but the subsequent metabolism of liberated fatty acids.

Free fatty acids are readily oxidized within cells in a process which may generate free radicals (Jones & Round, 1990). It seems that free - radical- mediated damaging process by intracellular Ca 2+ overload may be activated in skeletal muscle following treatment with a metabolic poison such as 2, 4-DNP. Reactive free radicals produce damage to proteins, lipids, carbohydrates and nucleotides and produce metabolic and cellular disturbances in vitro. They may also cause damage to cells through a pathway dependent essentially on membrane damage: - by covalent binding of free radicals to membrane enzymes and receptors, thus modifying the activities of membrane components, thereby altering the structure and affecting membrane function and/or antigenic character. Also, the disturbance of transport proteins; may be initiated by lipid peroxidation of polyunsaturated fatty acids with direct effects on membrane structure, and associated influences of the products of peroxidation on membrane fluidity, cross-linking, structure and function (Slater, 1984) Highly reactive free radicals are generally confined to their immediate vicinity as a result of their highly reactive nature. Consequently, their average diffusion radius is very small (Slater, 1984). This fact may provide a possible explanation for the adjacent areas of both stained and unstained cells as seen in the third experiment.

In conclusion, there appear to be three aspects of mechanisms causing damage associated with metabolite depletion:- a) calcium entry consequent upon change in muscle

metabolite levels e.g. decrease in levels of ATP; b) intracellular action of Ca 2+ in stimulating phospholipase, therefore causing damage to cellular membranes; c) free radical damage initiated by oxidation of liberated free fatty acids.

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تأثير حمل الكالسيوم وأضافة العقار ٢-٤ ثنائى نترات الفينول على النفاذيه الغشائيه للعضلات الهيكليه فى الفأر الابيض .

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أجريت هذه الدراسة لمعرفة تأثير العقار ٢-٤ ثنائى نترات الفينول على النفاذية الغشائيه للعضلات الهيكليه (عضلة الحجاب الحاجز) فى الفأر الابيض .

عند تحضين العضلة فى محلول العقار (ثنائى نترات الفينول عند تركيز ٢×١٠^{-٥} لمدة ١٢٠ دقيقه أحدث تأثير طفيف على نفاذية الغشاء البلازمى (والتى حسبت على درجة تغلغل الصبغ الأورانجى النشيط داخل الغشاء البلازمى للعضله) ثم زيادة التركيز بنقل العضلة لتركيز أعلى من العقار (٢-٤ ثنائى نترات الفينول الى تركيز ٢×١٠^{-٤}) لمدة ١٢٠ دقيقة أدت الى زيادة نفاذية الغشاء البلازمى فى حوالى ٢٨,٣% من مجاميع الخلايه التى فحصت وانحصرت النفاذية الغشائية فى الخلايا الطرفية الواقعة فى أطراف الغشاء البلازمى.

إزالة الكالسيوم من المحاليل الملحية نتج عنه أن النفاذية كانت فى حدود ٨% من مجاميع الخلايا الصابغة مقارنة ب ٢٨,٣% فى التجربة الثالثة (٢-٤ ثنائى الفينول لمدة ١٢٠) . هذه الزيادة فى مستوى النفاذية غشاء يمكن ان نعزوها للضرر الذى احدثه العقار ٢-٤ ثنائى نترات الفينول وحده ونستنتج ان الكالسيوم الخلوى الخارجى يلعب دورا مهما فى تحطيم الخلايا.