CHANGES IN PROTEIN PHOSPHORYLATION
DURING CHANGES IN VASCULAR
SMOOTH MUSCLE TONE

by

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We accept this thesis as conforming
to the required standard

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ABSTRACT

It is now generally accepted that changes in protein phosphorylation play an important role in mediating smooth muscle tone. An attempt was made to determine whether nitrogen oxide containing vasodilators such as sodium nitroprusside and nitroglycerin exert their relaxant effect via a phosphorylation reaction. Isoelectric focusing was initially used as a method of detecting phosphorylation. However, due to the smaller percentage of protein in smooth muscle with a relative increase in other cell constituents, crude smooth muscle homogenates were deemed to be too complex for analysis by this technique alone.

Phosphorylation changes are commonly studied by incubating the muscle in labelled inorganic phosphate, thus labelling the ATP pools. Proteins are separated by SDS-polyacrylamide gel electrophoresis. Following staining and drying, gels are exposed to X-ray film and phosphorylation levels determined.

By comparing aortic strips relaxed in Ca\textsuperscript{2+}-free, 5 mM EGTA Krebs with muscle strips contracted in K\textsuperscript{+}(124 mM) Krebs, a significant difference between the phosphorylation levels of myosin light chain was quantitated. By reproducing this well documented phenomena, we demonstrated that we had established a working methodology in the laboratory.

In view of the controversy in the literature concerning sustained levels of myosin light chain phosphorylation during sustained K\textsuperscript{+}-induced contractions, a K\textsuperscript{+}-time course study was performed. Levels of myosin light chain phosphorylation increased significantly within the first 2 min of contraction and were maintained for 12 min, though a non-significant decrease was observed after 2 min. Tension peaked at 4 min and thereafter
remained constant.

Finally, the effect of nitroglycerin ($10^{-M}$) on $K^+$-induced contractions was briefly examined. Nitroglycerin caused a 70% relaxation within 2 min and significantly decreased myosin light chain phosphorylation levels. There also appeared to be an increase in phosphorylation of a 160kD protein with nitroglycerin treatment, which due to technical difficulties could not be quantitated.
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LIST OF ABBREVIATIONS

ATP  adenosine 5'-triphosphate
ATPase  adenosine triphosphatase
cAMP  cyclic adenosine 5'-monophosphate
cGMP  cyclic guanosine 5'-monophosphate
EGTA  ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetra-acetic acid
IEF  isoelectric focusing
MLC  myosin light chain
MLCK  myosin light chain kinase
MLCP  myosin light chain phosphatase
NG  nitroglycerin
SDS-PAGE  sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SNP  sodium nitroprusside
TA  tibialis anterior
TCA  trichloroacetic acid
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INTRODUCTION

The calcium-dependent regulatory systems for the contractile proteins in smooth muscle have become the subject of intense research. The release of Ca\(^{++}\) into the sarcoplasm is the primary event in excitation-contraction coupling in all types of muscles. The subsequent binding of Ca\(^{++}\) to specific high-affinity sites on proteins associated with, or acting on, the contractile apparatus ultimately results in contraction, which is, in all types of muscle, the interaction of actin and myosin. The difference between muscles (i.e. skeletal and smooth) is the mechanism by which calcium causes this interaction. Such differences are attributed to differences in regulatory proteins, which mediate the Ca\(^{++}\) effects and to isozymic differences in the myosin molecule, (Stull et al, 1980).

One of the Ca\(^{++}\)-dependent processes that has been the subject of much study is the obligatory initiation of the actin-activated ATPase activity of myosin via the phosphorylation of the myosin light chain (MLC). This reaction is catalyzed by a calcium dependent myosin light chain kinase (MLCK), (Aksoy et al, 1976; Chacko et al, 1977; DiSalvo et al, 1978; Gorecka et al, 1976).

These studies have resulted in the hypothesis (Adelstein et al, 1977; Hartshorne et al, 1977) that calcium plays an important role in smooth muscle contraction by activating the MLCK, which in turn phosphorylates the MLC, which initiates an interaction between actin and myosin, resulting in contraction. According to the hypothesis, it would follow that a decrease in cytoplasmic calcium would inactivate MLCK and the MLC would be dephosphorylated by an endogenous myosin light chain phosphatase (MLCP) or phosphatases, resulting in relaxation.
The above hypothesis based on biochemical data from isolated protein studies is not unchallenged. (Mikawa et al, 1977) The main evidence against phosphorylation as the principal control mechanism for contraction is the apparent dissociation between phosphorylation and actin activated Mg-ATPase found by some workers. Studies by Mikawa et al, (1977), using smooth muscle actomyosin failed to detect any change in the activity of actomyosin ATPase with varying degrees of phosphorylation. Bose et al, (1979), using chicken gizzard actomyosin examined its calcium sensitivity. The authors concluded from this work that calcium sensitivity was altered by ATP concentrations, which appeared to regulate the degree of repression of myosin. These authors believe that at high levels of ATP myosin is repressed and can only interact with actin once it is phosphorylated. At low levels of ATP myosin is derepressed. In this state myosin can interact with actin as long as Mg is present and subsequently develop tension.

Alternative hypotheses proposed, include calcium binding to myosin, a new type of thin filament control system, which was named leiotonin and a troponin control (Marston, 1982) Inspite of these controversies, most investigators believe that phosphorylation of MLC is the main regulatory mechanism of contraction in smooth muscle. In addition to biochemical data obtained on studies in isolated proteins, correlations between increased levels of phosphorylation have been found in intact (Barron et al, 1979; Driska et al, 1979; Gualtieri et al, 1977) and functionally skinned (Hoar et al, 1979) smooth muscle preparations.

I.Evidence for a Role of Protein Phosphorylation in the Regulation of Smooth Muscle Tone
In reviewing some of the evidence for a role of light chain phosphorylation in smooth muscle regulation, it seems logical to first discuss the basal level of phosphorylation in a resting muscle, from there moving on to a spontaneously contracting muscle (e.g. myometrium) then to chemically-induced contractions and finally completing the cycle with a look at relaxation and relaxants. This is by no means a complete review of the literature in these areas but some of the major theories are highlighted, and some of the problems and differences in interpreting data are discussed.

a) Resting tension:

There is general agreement in the literature that even a resting muscle has detectable levels of MLC phosphorylation. This can be attributed to a number of possible reasons. When studies are carried out on intact smooth muscles, the muscles are generally under some degree of resting tension, the magnitude of which varies between types of muscles and species origin. Barron et al, (1979), demonstrated, using intact pig carotid arteries, that MLC phosphorylation was dependent upon the passive tension applied to the muscle. A similar observation was made by Dillon et al, (1981), using pig carotid arteries and de Lanerolle and Stull, (1980), using bovine trachea. It was proposed that stretching the muscle might cause an increase in cytoplasmic calcium concentration, which in turn might cause partial phosphorylation, (de Lanerolle and Stull, 1980). The degree of such phosphorylation might be commensurate with the tension of the muscle.

A second possible reason for the detectable levels of MLC phosphorylation in resting muscles was proposed by Aksoy et al, (1982). These authors suggested that there may be a slight artifactual increase in
MLC phosphorylation associated with the immersion freezing technique, however it can be argued that this is due to prevention of dephosphorylation which may otherwise occur in some cases during homogenisation due to incomplete inactivation of phosphatases.

Thirdly, it is conceivable that in muscles exhibiting spontaneous electrical activity (e.g. myometrium) this electrical activity is associated with a small influx of Ca$^{++}$, which could be sufficient to cause partial MLC phosphorylation. The exact origin of Ca$^{++}$ required for such a reaction in resting muscle is unclear.

Finally it has been suggested that a small (15%) systemic overestimation of phosphorylation will arise if isoelctric focusing (IEF) is used as a method of detection. This results from the carbamylation of the polypeptide, which will increase its negative charge - this is a possible result of tissue preparation. Other possible chemical modifications which may occur include oxidation, sulphonation of tyrosine residues, acetylation of lysine or N-terminal amino acids or deamination by isocyanates in urea, (Dillon et al, 1981). The use of IEF as a method of detection of protein phosphorylation stems from the observation that when a protein undergoes phosphorylation, its isoelctric point becomes significantly more acidic and this shift is detectable by IEF. Densitometry of the IEF gels is used to measure the changes, (Driska et al, 1981).

b) Spontaneous contractions:

In a study by Janis et al, (1980), using rat myometrium, these workers specifically looked at the levels of MLC phosphorylation during spontaneous contractions. Both one and two dimensional electrophoresis were used to determine phosphorylation and the results were presented as %
phosphorylation of relaxed controls, (Fig 1). These authors concluded that the results obtained support the hypothesis that contraction and relaxation of smooth muscle is partially regulated by phosphorylation and dephosphorylation of MLC. It also was observed that phosphorylation preceded maximal spontaneous contraction and dephosphorylation preceded maximal relaxation.

c) Induced contractions:

Not all smooth muscles will contract spontaneously and many studies have been carried out on induced contractions. Contractions may be initiated by a number of agonists acting at a variety of sites. In general, most studies agree with the hypothesis that MLC phosphorylation will precede contraction and dephosphorylation will precede relaxation.

Barron et al, (1980), demonstrated reversible MLC phosphorylation in swine carotid arteries, during the contraction-relaxation-contraction cycle using 100 mM KCl to contract the tissue and 10 mM theophylline to induce relaxation. The muscles were frozen after maximal tension (6 min treatment time) had been reached and there was an increase in phosphorylation in resting muscles from 0.48 mol phosphate / mol MLC to 0.65 mol / mol in contracted muscles. This level of MLC phosphorylation decreased to 0.31 mol / mol after 10 mM theophylline had caused complete relaxation (total treatment time 36 min). A similar phosphorylation of the MLC using noradrenaline as the contractile agonist had also been noted by these workers, (Barron et al, 1979).

However, in contrast to these results, Murphy and co-workers have suggested that phosphorylation declined significantly from its peak values before steady-state force was attained. Levels continued to decline towards
FIGURE 1:

Mechanical response of spontaneously contracting rat myometrium. Contractions occurred about one per minute. Also shown are the means for isotopic phosphorus incorporation at certain levels of isometric tension.

Reproduced from Janis et al. (1980).
P INCORPORATION INTO 20,000 M PROTEIN
% OF RELAXED CONTROL

ISOMETRIC TENSION (g)

TIME (sec)
control values after 10 min of stimulation. The contractile agonist in this case was 124 mM KCl and swine carotid arteries were the tissue used, (Driska et al, 1981). Peak MLC phosphorylation, with K⁺-stimulation was about 65% and occurred about 30 s after the muscle was exposed to the stimulus. Active force subsequently increased and was maintained while the levels of MLC phosphorylation were falling. After 12 min of stimulation the level of MLC phosphorylation was approximately 30%, (Fig 2).

The difference in the time course of MLC phosphorylation is not restricted to swine carotid arteries. A study by de Lanerolle and Stull, (1980), investigated MLC phosphorylation in canine tracheal smooth muscle and showed that the phosphorylation increased from a value of 0.50 to 1.1 mol phosphate / mol myosin, within 3 min after the addition of 100 μM methacholine. These authors concluded that MLC phosphorylation coincided temporally with the increase in isometric tension. Aksoy et al, (1982), attributed the thickness of the muscle preparation and the long agonist diffusion times as reasons for the failure to observe any rapid transient changes in these studies.

For the formentioned reasons, Gerthoffer and Murphy, (1983 a), used rabbit trachealis to study MLC phosphorylation. Results from their time course studies, using 10⁻⁵ M carbachol as a contractile agonist, were similar to those Driska et al, (1981), had found with swine carotid arteries. MLC phosphorylation was low in resting muscle, increased rapidly to 0.46 mol phosphate / mol MLC and subsequently declined toward resting levels prior to reaching steady-state active stress.

Silver and Stull, (1982), also found a transient increase in MLC phosphorylation in a thin preparation of bovine tracheal muscle. However, Janis et al, (1980), found that phosphorylation levels in rat uterine
FIGURE 2:

Relationship between developed force and MLC phosphorylation in strips prepared from the hog carotid media. A: open circles represent data from tissues frozen in the unstimulated state possessing low levels of tonic contractile activity. The remaining points were obtained from tissues frozen during a contraction produced by a high K\(^+\) physiological salt solution. Phosphorylation increased rapidly during the first 60s of the response, reaching a maximum value when force had reached about half of its maximum level (solid circles). After 2 min of stimulation, force was near maximum, although phosphorylation levels averaged somewhat lower (solid triangles). Strips frozen after approximately 6 min (open squares) or 12 min of stimulation (solid squares) showed progressively lower levels of MLC phosphorylation. Maximum active force (±SE) averaged 3.34±0.15x10^5 n/m^2 (n=17), and, once developed, was maintained without significant changes in the presence of high K\(^+\).

Reproduced from Driska et al. (1981)
A Contracting Tissues

% Phosphorylation (LC 20P/LC 20U+LC 20P)/100

Active Force (% of Maximum)
smooth muscle were still elevated at 0.72 mol phosphate / mol MLC after 3 - 5 min 100 µM carbachol. Resting levels were estimated at 0.4 mol / mol.

There appear to be two schools of thought on the role of MLC phosphorylation, one that it is associated with contraction and is maintained during contraction and the other that it is more closely related to isotonic shortening velocities and cross-bridge cycling rates, (Aksoy et al, 1982; Dillon et al, 1981).

d) Calcium dependency:

Calcium has long been implicated as a regulatory ion in muscle contraction. There are many examples in the literature showing a correlation between calcium concentration, tension and MLC phosphorylation.

In the following example, (Chatterjee et al, 1983), skinned carotid arteries were used to study the calcium requirements of MLC phosphorylation, (Fig 3). As the concentration of calcium increased, the MLC phosphorylation and tension increased. If calcium is omitted from the physiological salt solution and 1.0 mM EGTA added, there is a marked decrease in the MLC phosphorylation, (Table 1), (Barron et al., 1979). Also shown in Table 1 is the effect of passive tension on phosphorylation as was previously discussed.

It has been suggested that maximum phosphorylation will occur at calcium concentrations of 10^{-5} M, (Driska et al, 1981). However, Murphy and co-workers over the past few years, have produced a number of reports that suggest that contraction can be maintained without phosphorylation, (Chatterjee et al, 1983; Dillon et al, 1981; Aksoy et al, 1982; Aksoy et al, 1983).

These workers summarise the hypothesis for the contraction-relaxation
FIGURE 3:

Steady-state Ca$^{2+}$ dependence of stress and myosin phosphorylation when Ca$^{2+}$ was increased from 1.8x10^{-8} M (means±standard errors, n=4 to 6). Maximum stress was 9.3±1.2x10^{4} n/m^{2} (n=10). The calculated concentration of Ca$^{2+}$ for half-maximal change in the response ($K_{50}$) is shown with a 65% confidence interval (■).

Reproduced from Chatterjee et al. (1983).
Increasing Ca

Percent maximum stress (%)

Mole of P per mole of LC 20 (o)

pCa

6.7 x 10^{-7}

6.4 x 10^{-7}
**TABLE 1:**

Phosphorylation of 20,000 dalton LC of myosin in intact arterial smooth muscle under various conditions.

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<th>Condition</th>
<th>$^{32}\text{Phosphate/MLC}$ mol/mol</th>
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<tr>
<td>Resting, with passive tension $\text{Ca}^{2+}$-free soln*</td>
<td>0.13</td>
</tr>
<tr>
<td>Resting, with no passive tension and $\text{Ca}^{2+}$</td>
<td>0.34</td>
</tr>
<tr>
<td>Resting, with passive tension and $\text{Ca}^{2+}$</td>
<td>0.55</td>
</tr>
<tr>
<td>Contracted, with norepinephrine</td>
<td>0.79</td>
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* $\text{Ca}^{2+}$ was omitted from the physiological salt solution and 1.0 mM EGTA was added.

Barron et al., 1979
cycle for arterial smooth muscle as follows, (see Fig 4). Relaxed muscle contains thin actin filaments (A) and unattached myosin cross-bridges (M). A rise in calcium concentration on stimulation activates MLCK and phosphorylates free cross-bridges (M-P), which then can interact with actin. Evidence indicates that kinase-phosphatase systems can also act on attached cross-bridges. Dephosphorylation of attached cycling cross-bridges (AM-P) arrests cycling to form attached, noncycling cross-bridges (AM), termed latch bridges. Latch bridges constitute internal load on cycling cross-bridges and decrease shortening velocity (heavy arrow). Second, direct action of calcium on thin filaments or myosin is shown by dependence of stress (reflecting both AM and AM-P) on this ion. Dashed arrows indicate possibilities that detachment of latch bridges may occur by mechanical breakage and that direct formation may occur in the presence of calcium, (Aksoy et al, 1982).

These latch bridges could explain why arteries, which have a comparable energy usage to that of skeletal muscle during development of isometric tension, can maintain high degrees of tone inspite of a subsequent decrease in energy usage during steady-state maintenance of tone, (Aksoy et al, 1982).

e) Relaxation:

As has been previously discussed, MLC phosphorylation is reversible and dephosphorylation is often associated with relaxation, (Janis et al, 1980; Barron et al, 1980; Chatterjee et al, 1983; Aksoy et al, 1982; Driska et al, 1981).

However, Murphy and coworkers have produced evidence that phosphorylation is not necessarily sustained during contraction and it
FIGURE 4:

Hypothesis for regulation of cross bridges.

See text for explanation.

Reproduced from Aksoy et al. (1982).
would follow that dephosphorylation might then not be so strictly correlated with relaxation. Therefore Gerthoffer and Murphy, (1983 b), examined the relationship between dephosphorylation and relaxation.

Using intact swine carotid arteries, the above authors contracted the tissue with 110 mM KCl solution and then washed the agonist out. The relaxation following washout was analyzed as a dual exponential decay. The initial rapid phase (2 min) was associated with dephosphorylation and the decay capacity of isotonic shortening velocity. The second slow phase (45 min) was attributed to the noncycling latch bridges. The rate of decay of this slow phase was enhanced by the removal of calcium and retarded by an increase in calcium to 5 mM. These results tend to support the above hypothesis as proposed by Aksoy et al, (1982), (see Fig 4), illustrating the existence of two types of cross-bridges, one population of which is dephosphorylated, sensitive to calcium and capable of maintaining stress.

Taking this one step further, how do relaxants affect phosphorylation levels? Since the discovery by Perry, (1979), of the MLCK / MLCP system, a growing body of evidence has strongly implicated MLC phosphorylation in the calcium-dependent regulation of contraction of smooth muscle. This is not the only enzyme system in operation. Adelstein and Hathaway, (1979), described a system, which is dependent on cyclic AMP (cAMP). The cAMP-dependent protein kinase will phosphorylate MLCK, decreasing its affinity for the Ca\textsuperscript{++}-calmodulin complex thus decreasing levels of MLC phosphorylation. This led Adelstein and Hathaway to postulate that increased cAMP levels would result in phosphorylation of MLCK, a decrease in phosphorylation of MLC and relaxation. These workers proposed that beta-adrenergic agonists, which cause relaxation of contracted vascular smooth muscle, exerted their effects in this way.
Considering the theory presented by Murphy and colleagues concerning latch bridges, (Fig 4), (Aksoy et al, 1982; Gerthoffer and Murphy, 1983 a, b), the above hypothesis proposed by Adelstein and Hathaway, (1979), seems lacking. How can relaxants induce relaxation via MLC dephosphorylation if phosphorylation is, as studies by Murphy and coworkers suggest, already at basal levels? A recent investigation by Gerthoffer and Murphy (in press) addresses this very question.

Using swine carotid arteries as previously described, (Driska et al, 1981), these workers examined the effects of various relaxants on K⁺- and phenylephrine-induced contractions. Muscles were contracted for 15 min, after which the agonist was washed out and the relaxant added 2 min later, when phosphorylation levels were near control values, (Gerthoffer and Murphy, 1983 b). The relaxants chosen represented a variety of different classes, adenosine and forskolin, which are thought to act via cAMP-dependent mechanisms, 3-isobutyl-1 methyl xanthine as a phosphodiesterase inhibitor and sodium nitroprusside (SNP) and 8-bromo-cGMP, which are thought to act via cyclic GMP (cGMP) dependent mechanisms. In all cases the results were similar. The above agents all enhanced the relaxation rate after MLC had been dephosphorylated.

These results support the hypothesis, (Aksoy et al, 1982), (see Fig 4), that there are two regulatory mechanisms governing vascular smooth muscle tone.
1) Initial phosphorylation of MLC which results in phosphorylated cycling cross bridges.
2) Following dephosphorylation of the above, stress is maintained by non-cycling cross-bridges termed latch bridges, which are calcium dependent in an as yet unknown manner.
Cyclic AMP-induced dephosphorylation by beta adrenergic agonists would not appear to be the primary method of relaxation by these agents.

f) Vasodilators:

In the last section, various vasodilators were mentioned as well as their effects on cyclic nucleotides. The relationship between cyclic nucleotides, particularly cGMP, and mechanical activity of smooth muscle has been the subject of several disparate theories. Increases, (Lee et al, 1972), as well as decreases in smooth muscle tension, (Diamond and Holmes, 1975), were observed to be associated with increased cGMP levels. Subsequently, it was found that a number of clinically useful vasodilators, including nitroglycerin (NG) and SNP, could increase cGMP levels in the absence of Ca²⁺ions in intact smooth muscle tissue, (Schultz et al, 1977). The hypothesis, which is becoming widely accepted, was then put forward that these nitrogen oxide containing compounds exert their relaxant effect by virtue of their ability to increase levels of cGMP in vascular smooth muscle.

There are many observations in the literature consistent with this hypothesis. A variety of smooth muscle relaxants have been shown to increase levels of cGMP in various tissues, (Katsuki and Murad, 1977; Katsuki et al, 1977 a, b; Bohme et al, 1978; Janis and Diamond, 1979). Some of these vasodilators, namely NG and SNP, also activate guanylate cyclase directly, (Gruetter et al, 1980, 1981). There appears to be a close correlation between the degree of smooth muscle relaxation and the extent of cGMP formation by NG, SNP and other smooth muscle relaxing agents, (Katsuki and Murad, 1977; Axelsson et al, 1979; Janis and Diamond, 1979; Kukovetz et al, 1979). Another piece of evidence in favour of the
hypothesis is that the lipophilic derivative of cGMP, 8Br-cGMP, is capable of relaxing smooth muscle, (Katsuki and Murad, 1977; Schultz et al, 1979; Napoli et al, 1980).

Studies by Gruetter et al, (1981), show that cGMP accumulation actually preceded the onset of relaxation induced by NG and was temporally correlated with relaxation induced by NG and SNP. This study also demonstrated that an inhibitor of guanylate cyclase, methylene blue, could simultaneously inhibit cGMP accumulation and relaxation by nitrogen oxide-containing vasodilators. The return of cGMP levels to control values preceded the return of arterial tone. These results were later confirmed by Keith et al, (1982). Furthermore, inhibition of vascular smooth muscle relaxation was seen with NG tolerance and there was a corresponding inhibition of cGMP generation, (Keith et al, 1982). Subsequent action of cGMP does not appear to be impaired by the presence of NG tolerance or the inhibitor, methylene blue, since 8Br-cGMP is still able to relax vascular smooth muscle. This evidence would tend to suggest that the ability to generate cGMP may be an important step in the process by which NG and SNP relax vascular smooth muscle.

It is thought that these nitrogen oxide containing compounds relax vascular smooth muscle via the reactive intermediate, nitric oxide (NO) and that the biological action of NO is associated with the activation of guanylate cyclase, (Gruetter et al, 1979). This would stimulate cGMP formation in vascular and non vascular smooth muscle, (Schultz et al, 1977; Katsuki et al, 1977 a, b). These increases in cGMP may then initiate relaxation in some, as yet unknown, way.

Despite all the above evidence, results from this laboratory are not consistent with the hypothesis that cGMP and relaxation are causally
related at least in some types of smooth muscle. SNP was able to produce large increases in cGMP levels in K⁺- or phenylephrine-contracted strips of rat vas deferens without causing relaxation, while hydralazine and verapamil caused relaxation without an increase in cGMP levels, (Diamond and Janis, 1978).

When other types of smooth muscle, such as guinea-pig taenia coli and vascular smooth muscle were examined, the results indicated that in these tissues also, the relaxant effects of hydralazine and verapamil were not associated with increased cGMP levels. Low concentrations of SNP were also able to relax taenia coli without simultaneously elevating cGMP levels, however at concentrations greater than 1 μM there appeared to be a correlation between relaxation and increases in cGMP, (Janis and Diamond, 1979).

In more recent experiments from this laboratory it was shown that both NG and SNP are able to cause an elevation in cGMP levels in the rat vas deferens and myometrium, though only NG can cause relaxation, despite the fact that the increase in cGMP levels was greater with SNP, (Diamond, 1983).

This data still might be compatible with the hypothesis if these tissues do not possess a cGMP-dependent mechanism of relaxation and therefore do not relax on increases in cGMP levels caused by such compounds as SNP. NG, however may have two mechanisms of relaxation (one cGMP-dependent and one cGMP-independent) and, at high concentrations of NG the latter mechanism could be responsible for the relaxation observed.

However, as pointed out earlier, the exogenous administration of 8Br-cGMP was capable of relaxing these preparations and is cited as strong evidence in favour of the proposed role for cGMP as a mediator of smooth
muscle relaxation, (Schultz et al, 1979; Napoli et al, 1980). If 8Br-cGMP can relax these muscles, presumably by increasing tissue levels of cGMP, why are the increases in cGMP levels caused by SNP not accompanied by relaxation? It is possible that in these tissues cGMP does not play a role in relaxation and that 8Br-cGMP can relax the muscle by a mechanism independent of cGMP.

Alternatively, the increases in cGMP might occur in a compartment or pool which is not physiologically relevant. For example, it is thought that cGMP exerts its effect via activation of a cGMP-dependent protein kinase. If this is a necessary step in smooth muscle relaxation it is possible that in some tissues this kinase is inaccessible to the increased cGMP levels due to compartmentalization of the latter.

Preliminary observations from this laboratory suggest that smooth muscle relaxation and activation of cGMP-dependent protein kinase may be correlated under certain conditions. The activity of the kinase is difficult to measure directly, due to technical problems. If however, activation of cGMP-dependent protein kinase is involved in mediating vascular relaxation, a possible mechanistic pathway is via phosphorylation of specific protein(s), which are involved in the regulation of smooth muscle tension in the muscle. Reports in the literature, (Draznin et al, 1982, Rapoport et al, 1982), have shown increased phosphorylation of several proteins in rat aortic tissues exposed to 0.5 μM SNP for 2-15 min. These authors suggested that one or more of these proteins may be involved in the SNP induced relaxation of vascular smooth muscle.
OBJECTIVES

1) The main objective of this project was to establish a working protocol in our laboratory for measuring protein phosphorylation in smooth muscle. As a measure of the suitability of the method we attempted to quantitate phosphorylation of MLC during calcium-induced contractions of rabbit aorta. Calcium-dependent phosphorylation of MLC in smooth muscle is a well documented phenomenon.

2) A second objective was to examine the time course of MLC phosphorylation during potassium-induced contractions of rabbit aorta.

3) A third objective was to demonstrate changes in protein phosphorylation during relaxation of potassium-contracted arteries by nitroglycerin.
MATERIALS AND METHODS

A) MATERIALS:

1) Animals:

Female Wistar rats were oestrogen primed 24hrs prior to sacrifice. They were killed by cervical dislocation and two myometrial strips were prepared from each animal.

White New Zealand rabbits of either sex (2 - 3kg) were killed by a blow to the base of the skull and bled. Descending thoracic aortas were then removed.

2) Chemicals:

i) Radioisotopes

$^{32}$P as carrier free H$_3$PO$_4$ (285 Ci / mg at 100% isotopic enrichment) was obtained from ICN.

ii) Electrophoresis:

Sucrose and acetic acid were obtained from Analar. The following were purchased from Bio-Rad: ammonium persulphate, bisacrylamide, SDS-PAGE high and low molecular weight standards. Ampholines pH range 5-7 and 3.5-10 were purchased from LKB and Bio-Rad. Acrylamide and SDS were initially purchased from Sigma chemical Co. and then Bio-Rad was used as the supplier due to increased purity. Phosphoric acid and sodium hydroxide were purchased from Fisher Scientific. Urea was purchased from Mallinckrodt. Ethanol and methanol were purchased from T and B Westline. All other reagents for electrophoresis were purchased from Sigma Chemical Co.

iii) Other reagents:

Sodium molybdate was purchased from Allied Chemical Baker and Adamson.
Isopropyl acetate, sodium dihyrogen phosphate and sodium hydrogen carbonate were purchased from Analar. The following were purchased from Amachem: Calcium chloride, glucose, potassium chloride, potassium dihydrogen phosphate. Aqueous counting scintillation fluid and econfluor were purchased from Amersham Radiochemicals. Perchloric acid was purchased from G. Fredrick Smith Chemical Co. Magnesium sulphate and 2-methyl butane were purchased from MCB Manufacturing Chemists. Nitroglycerin was purchased from Lilly. All other reagents were purchased from Sigma Chemical Co.
B) METHODS

1) Tissue preparation:

The initial studies involving IEF utilised rat myometrium. Female Wistar rats (250 - 300 g) were oestrogen primed 24 hours before sacrifice with a subcutaneous injection of 50 μg estradiol benzoate (dissolved in peanut oil). Rats were killed by cervical dislocation and two myometrial strips were prepared from each animal as described by Diamond and Hartle, (1974). The incubation solution was (in mM): NaCl, 125; KCl, 2.4; MgCl₂, 0.5; CaCl₂, 1.8; glucose, 11; and Tris-HCl, 23.8 (pH 7.0). The solution was aerated with 95% O₂/ 5% CO₂ and maintained at 37°C. Strips were incubated for 30 min with a resting tension of 0.5 g. Isoproterenol was added directly to the baths and the muscles were frozen at predetermined times.

Studies involving SDS polyacrylamide gel electrophoresis (SDS-PAGE) were conducted on rabbit aorta. Descending thoracic aortae were removed from white New Zealand rabbits of either sex (2 - 3 kg) and were stripped of superficial fat. Helical strips, approximately 3 x 10 mm, were prepared and suspended in 3 ml organ baths between stainless steel hooks for recording of isometric tension. Tension was monitored on a Grass model 7C polygraph. The strips were equilibrated for 30 min at 37°C in a physiological salt solution with the following composition (mM): NaCl, 118; KCl, 5.7; MgSO₄, 2.37; CaCl₂, 1.26; NaH₂PO₄, 1.17; NaHCO₃, 25 and glucose, 11. Preparations were oxygenated by bubbling with 95% O₂/ 5% CO₂, which maintained the pH of the solution at 7.4. A resting tension of 2 g was applied to the strip and readjusted frequently throughout the equilibration period.

200 μCi of carrier free ³²P was then added directly to each bath and
the strips were labelled for a period of 60 min. The $^{32}P$ in the extracellular space was removed by a series of at least five rinses. Muscles strips were treated as indicated in the results and nitroglycerin was added directly to the baths to give a final concentration of $10^{-6}$ M. Aortic strips were frozen at appropriate times after drug addition by means of a Wollenberger-type clamp precooled to -80°C.

2. Sample preparation:

The methods used in preparing samples are discussed in detail in the results with the IEF gels produced.

Samples were prepared for SDS-PAGE by the method of Janis et al., (1980). Frozen muscles were homogenised using an Eberbach homogeniser with a tight fitting pestle, in 1 ml ice cold 12% TCA in water. The pestle was then rinsed in 1 ml 12% TCA. The homogenates were centrifuged at 6,000 x G for 10 min. The acid was extracted from the pellet with ether. The pellet was redissolved in a sample buffer, comprising 10% (w/v) SDS / 125 mM Tris/HCl (pH 6.8) / 20% (w/v) sucrose / 150 mM 2-mercaptoethanol. The resulting suspension was boiled for 5 min. Bromophenol blue was added to each sample and the sample respun at 6000 x G for 5 min. Aliquots containing approximately 150 mg protein were loaded onto SDS slab gels.

3. Electrophoretic techniques:

a) IEF

Solutions:

Lysis buffer: 9.5 M urea, 2% w/v NP-40, 2% Ampholines (comprised of 1.6% pH range 5 to 7 and 0.4% pH range 3.5 to 10) and 5% beta mercaptoethanol - stored as frozen aliquots. Gel overlay solution: 8 mM
urea. Sample overlay solution: 9 M urea, 1% Ampholines (comprising of 0.8% pH 5 to 7 and 0.2% pH 3.5 to 10) - stored as frozen aliquots. Acrylamide stock solution: 7.1 g acrylamide and 0.405 g bisacrylamide in 25 ml distilled water, made fresh each time. 10% w/v NP-40 solution: 1 g / 10 ml was made up and stored at 4 C. Fixing solution: 150 ml methanol, 350 ml distilled water, 17.25 g sulphosalicylic acid, and 57.5 g trichloracetic acid. Destaining solution: 500 ml ethanol, 160 ml acetic acid, diluted to 2 L with distilled water.

Procedure:

IEF gels were made in glass tubing (130 mm x 5 mm inside diameter) according to the method of O'Farrell, (1975). The tubes were cleaned in chromic acid for 24 hrs, rinsed well in hot water and then soaked in 1N KOH / methanol for 24 hrs to neutralise the acid. They were subsequently rinsed thoroughly in hot water followed by distilled water. It was very important to ensure that the tubes were clean as this avoided the gels slipping out during a run and made extrusion of the gels easier. The tubes were dried in an oven and the ends sealed with Parafilm, while still warm as this ensured a good seal.

The gel mixture was made in a 125 ml flask from 22 g ultrapure, powdered urea, 5.2 ml acrylamide stock solution, 8 ml 10% NP-40 solution and 7.8 ml distilled water. The above were mixed for approximately 15 min and then given a short blast of heat (37°C) to aid dissolution of the urea. Since cyanate formation from urea is accelerated with increasing temperature, it was important not to overheat or the urea. Cyanate reacts with amino groups forming carbamylated derivatives thus altering the charge on the protein, (Hames, 1982). Subsequently, 1.6 ml Ampholine pH 5-7 (40% commerical preparation), 0.4 ml Ampholine pH 3.5-10 (40% commerical
preparation) were added slowly. When adding ampholites the cap of the vial was wiped with 95% ethanol before and after insertion of the needle to try to keep the solution reasonably sterile. 40 μl 10% fresh ammonium persulphate (2 mg / 2 ml) were added, followed by 20 μl TEMED. The gel mixture was stirred well, avoiding the incorporation of a lot of bubbles. The gel would not set in the flask due to the amount of O₂ that it was exposed to.

Using a long necked disposable pipette the tubes were carefully filled with gel mixture. Each tube had a notch at 120 mm and were filled past this mark and then enough gel was removed to drop the meniscus down to the notch. As the length of the gel affects the reproducibility, it was important to keep this measurement consistent between samples that were to be compared. Once all the gels were poured, some of the remaining gel mixture was drawn into the pipette and allowed to set. This was to determine if the gel mixture polymerised in the tubes.

The gel mixture was overlaid very carefully with 50 μl gel overlay solution and left to set for 1 - 2 hours. The gel overlay solution was removed and replaced with 100 μl fresh lysis buffer, which was overlaid with distilled water and left for 1 hour. The Parafilm was removed and the gels placed in a standard tube gel electrophoresis chamber. The fluid at the top of the gel was removed and 100 μl fresh lysis buffer added, this was overlaid with 0.02 M NaOH. The upper reservoir was filled with 0.02 M NaOH and the lower with 0.01 M phosphoric acid. Any bubbles at the bottom of the tubes were removed with the curved pipette. The gels were prerun according to the following schedule a) 200 volts for 1/4 hour b) 300 volts for 1/2 hour c) 400 volts for 1/2 hour.

The upper reservoir was emptied and the fluids removed from the top of
the gels. The samples were loaded and overlaid with 40 µl of sample overlay. This was overlaid with 0.02 M NaOH. The upper reservoir was refilled with NaOH. The samples were focused for 17 hours at 400 volts and 1 hour at 800 volts. There was enough acid in the lower reservoir to cover most of the length of the tube, this acted as a heat sink and prevented the gels overheating with the voltage.

Gels were fixed, stained and destained according to the method of Jasch et al., (1982). They were removed from the tubes by expulsion with air, directly into tubes (7 mm x 140 mm) containing a fixing solution for 2 hours, rinsed in destain for 10 min and then stained for 30 min in a 60°C water bath in a stain comprised of 0.115 mg Coomassie Brilliant Blue R 250 and 100 ml destain, (staining solution was filtered before use). After staining gels were rinsed in destain and transferred to 16 x 150 mm capped culture tubes filled with destain. After 3 days of destaining in the dark, the gels were transferred back into the 7 mm diameter tubes with fresh destain and on day 4 were scanned on a Gilford spectrophotometer, fitted with a Gilford gel scanner. Normally gels were scanned by adjusting the full scale absorbance or wavelength (from 560 nm to lower wavelengths) so that the highest peak of the scan reached the top of the chart paper. Gels were photographed on the day after scanning.

b) SDS-PAGE

Solutions:

Stain; 1.375 g Coomassie Brilliant Blue R 250, 500 ml methanol, 100 ml acetic acid and 500 ml distilled water. Destain I; 500 ml methanol, 500 ml distilled water and 100 ml acetic acid. Destain II; 100 ml acetic acid, 400 ml methanol and 1500 ml distilled water.
Procedure:

As for the IEF gels - the glass plates for casting slab gels had to be scrupulously cleaned before use, to avoid contamination of gels by exogenous proteins. The gels were cast according to the method of Laemmli and Favre, (1973). A Bio-Rad model 220 apparatus was used with a separating gel 180 mm wide, 120 mm high and 3 mm thick. Typically, 5-20% gradient gels were used as they gave better resolution of low molecular weight proteins than did 12% gels. Gradients were cast using a Pharmacia Gradient Mixer GM-1 and a LKB Varioperpex II peristaltic pump. No difference in resolution was found between gels which had been allowed to set overnight and those which were used within 3 hours of casting. Stacking gels were 40 mm high and consisted of 5% gel.

150 µg of protein was added per well and molecular weight standards were run concomitantly on each gel. The protein standards used for the estimation of molecular weight (in daltons) myosin (200,000), galactosides (116,250), phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400). The gels were run at a constant current of 60 mA, using a Pharmacia power supply (Model EPS 500/400), until the bromophenol blue marker dye had reached the bottom of the gel. Cooling was necessary. Gels were stained for 30 min, the stain poured off and reused. The gels were rinsed in water and gently agitated in destain I until destaining was completed and rehydrated in destain II. They were dried by heat under vacuum using a Bio-Rad model 224 slab gel dryer. The dried gel was placed in contact with X-ray film (Kodak Min-R), in some cases along with intensifier screens (Cronex Lightning Plus) for 7 - 30 days, following which, the film was developed. After both gels and autoradiograms had been
photographed, the autoradiograms were scanned at 560 nm in a Gelman DCD-16 gel scanner. The optical density was altered with each scan to ensure that the tallest peak was at the top of the chart paper.

4. Miscellaneous assays

a) Protein assay:

A protein assay was performed on the resuspended pellet before the bromophenol blue was added, by the method of Zaman and Verwilghen, (1979). It was a simple method devised for the determination of protein concentration in just such a solution as the one used above. The method was based on protein and Coomassie Brilliant Blue G-250 binding but it involves an initial step, which precipitates excess SDS with 100 mM potassium phosphate buffer, pH 7.4 to 7.5. Bovine serum albumin was used as a standard.

b) Statistics:

All data are presented as means ± S.D., (Glantz, 1980). Differences between treatments were tested using a student "t" test or in the case of the K⁺-time course study by a modified "t" test and the Bonferroni method of simultaneous multiple comparisons, (Wallenstein et al, 1980).
RESULTS

IEF

Sample preparation;

Preliminary experiments were carried out to determine the most suitable method of sample preparation for the type of muscle chosen - rat myometrium. The varieties of methods of sample preparation are as numerous as smooth muscle types to be studied.

Five different methods were selected and tube gels of each sample were run in duplicate as indicated in Methods. The critical step in sample preparation in this type of study is the immediate and complete inactivation of the kinase and phosphatase. If these enzymes are not denatured at the onset of sample preparation, alterations in phosphorylation levels will occur. A muscle sample containing a phosphorylated protein was the tibialis anterior (TA) muscle of the mouse, (Dr. L.G. Jasch, personal communication). Experiments assessing the effect of sample preparation on the levels of phosphorylation could than be performed using this muscle. (No previous studies had reported an IEF profile for rat myometrium. Thus, although this was the muscle of interest in this study it could not be used to assess sample preparative techniques.).

A. A previously reported method of mouse skeletal muscle sample preparation, (Jasch et al, 1982), involved homogenisation in Tris buffer (pH 7.4, 4°C). Aliquots of the homogenate containing the desired weight of muscle tissue, were withdrawn and lyophilised for 20 hours. Samples were rehydrated and partially solubilised in lysis buffer, (O'Farrell, 1975). The entire sample, which due to the high protein content was partially
gelled, was loaded onto the IEF gel. This method was used as a control. TA and myometrium were prepared in this manner.

B. Since Rapoport et al, (1982), had carried out similar studies on protein phosphorylation, their method of sample preparation was also used. This involved homogenisation in an ice-cold stopping buffer (100 mM NaF / 80 mM sucrose / 10 mM EDTA / 10 mM N\text{tris(hydroxymethyl)} methyl\text{aminoethanesulphonic acid, pH 7.4}, Garrison, 1978). NaF and EDTA were added as inhibitors of kinases and phosphatases. Homogenates were spun at 105,000 x G for 60 min. The supernatant fraction was removed and the pellet resuspended in lysis buffer as previously described. This would yield the particulate fraction only.

C. Samples were homogenised in stopping buffer as in method B. The resulting homogenate was lyophilised as in method A.

D. Janis et al, (1980), when preparing myometrial samples for 2-D electrophoresis used the method of Garrison, (1978), as described above (method B) but for one-dimensional electrophoresis the muscle was homogenised in TCA solution and the precipitated protein spun down at 6,000 x G for 10 min, as described in the methods.

E. Since the experimental protocol to be used would involve freezing the muscle, samples were first frozen, ground in TCA and prepared as described in D above.

F. A report appeared in the literature, (Driska et al, 1981), in which a study comparing the levels of phosphorylation in muscles, which had been freeze-clamped to samples which were frozen in partially frozen acetone slush. The frozen muscle samples were allowed to reach room temperature in the thawing acetone. These authors believe acetone would denature the endogenous enzymes and preserve levels of phosphorylation prevailing at the
time of freezing. The tissues were subsequently homogenised. Muscle samples were denatured in this manner and then homogenised in TCA solution as described for D.

Table 2 represents a summary of the methods used.

TA and myometrial strips were run in parallel. The degree of phosphorylation in TA samples was estimated from the ratio of peak height of the phosphorylated to non-phosphorylated protein. An alteration in this value was taken as an indication of the suitability of the method. Table 3 summarises the data from TA muscles and Fig 5 shows two of the scans obtained. From pilot studies it appeared that there was no significant differences between methods D and E and therefore method D was omitted from subsequent experiments, for this reason it does not appear in Table 3.

Comparing the values given in Table 3, it appears that inhibition of enzyme activity at the onset of sample preparation is necessary. Unlike the studies conducted by Driska et al., (1981), there did not appear to be any significant difference between freeze-clamping and fixing in acetone slush, each yielding a value of 77% phosphorylation. The best yields of phosphorylation appeared to be achieved when the stopping buffer of Garrison, (1978), was used, (83%). Unfortunately this method has disadvantages. If the sample is lyophilised with stopping buffer, (Fig 6, lower scan), there is a non-indentical shift in bands. When the sample is prepared as outlined by method B only the particulate fraction is used, eliminating the observation of possible changes in phosphorylation levels of proteins remaining in the soluble fraction. This point is clearly indicated by the myometrial samples depicted in Fig. 7. It can be seen by comparing the scans that certain peaks appearing towards the basic end of the lower scan B, are missing in the upper scan A.
# TABLE 2:

Summary of methods used for sample preparation prior to IEF.

<table>
<thead>
<tr>
<th>Method Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Homogenisation in 1 mM Tris, lyophilisation and reconstitution in lysis buffer</td>
<td>Jasch et al., 1982</td>
</tr>
<tr>
<td>B: Homogenisation in stopping buffer, centrifugation at 105,000 x G for 60 min and reconstitution of pellet in lysis buffer</td>
<td>Garrison et al., 1978</td>
</tr>
<tr>
<td>C: Homogenisation in stopping buffer, lyophilisation and reconstitution in lysis buffer</td>
<td></td>
</tr>
<tr>
<td>D: Homogenisation in 12% TCA, centrifugation at 6,000 x G for 10 min and reconstitution of pellet in lysis buffer</td>
<td>Janis et al., 1980</td>
</tr>
<tr>
<td>E: Freeze clamp muscle - prepare as in D</td>
<td>Driska et al., 1981</td>
</tr>
<tr>
<td>F: Denature in acetone slush - prepare as in D</td>
<td>Janis et al., 1980</td>
</tr>
</tbody>
</table>

Driska et al., 1981
TABLE 3:

% Phosphorylation of protein in tibialis anterior samples prepared by various methods.

<table>
<thead>
<tr>
<th>Method of preparation</th>
<th>% phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Homogenisation in 1 mM Tris lyophilisation</td>
<td>46</td>
</tr>
<tr>
<td>B: Homogenisation in stopping buffer, centrifugation at 105,000 x G for 1 hr</td>
<td>83</td>
</tr>
<tr>
<td>C: Homogenisation in stopping buffer, lyophilisation</td>
<td>81</td>
</tr>
<tr>
<td>E: Freeze clamp, homogenisation in 12% TCA, centrifugation at 6000 x G for 10 min</td>
<td>77</td>
</tr>
<tr>
<td>F: Fix in acetone slush, homogenisation in 12% TCA, centrifugation at 6000 x G for 10 min</td>
<td>77</td>
</tr>
</tbody>
</table>
FIGURE 5:

Comparison of the distribution of proteins as resolved on isoelectric focusing polyacrylamide gels in 6 week tibialis anterior samples; acidic end is to the right. Each gel contained proteins derived from the whole homogenate of 11 mg wet weight muscle. Approximately the top (at left) centimeter of each gel and all gels illustrated in subsequent plates was cut off to accommodate the gel in the 10 cm scanning cuvette.

A: Muscle was homogenised in 1 mM Tris (pH 7.4) as per method A in text.

B: Muscle was frozen, fixed in dry ice acetone and homogenised in 12% TCA as per method F in text. X And X-P represent a non-phosphorylated protein and its phosphorylated counterpart.
FIGURE 6:

Scans of 6 week tibialis anterior. Orientation and labels as in Figure 5.

A. Muscle was prepared by method A (see text).

B. Muscle was prepared by method E (see text).
FIGURE 7:

Comparison of the distribution of proteins as resolved on isoelectric focusing polyacrylamide gels in myometrial samples. Gels A & B contained proteins derived from the whole homogenate of 15 mg and 12.5 mg wet weight muscle respectively. Acidic end is on the left.

A: Myometrium was prepared by method B - see text.

B: Myometrium was prepared by method D - see text.
By virtue of the above pilot study, freeze-clamping and subsequent homogenisation in 12% TCA was chosen as a method of sample preparation.

Initially a comparison between relaxed controls and strips contracted with a submaximal dose (40 mM KCl) for 10 min was made. Spontaneously contracting myometrial strips were suspended and incubated in Tyrodes as described in the methods. Relaxed controls were muscles frozen in the relaxed state between spontaneous contractions. Contracted myometrium were treated with 40 mM KCl Tyrodes, in which NaCl had been replaced with an equimolar concentration of KCl to give the desired final KCl concentration, (40 mM KCl). Fig 8 shows typical scans of the tube gels obtained. No consistent significant changes could be seen.

In trying to prompt some kind of phosphorylation, K⁺-contracted strips, (10 min) were relaxed with isoproterenol 10⁻⁶ M, (1 min). Again there were no detectable changes in the scans (data not shown). Finally myometrial strips were relaxed in Ca²⁺ free Tyrodes and some were then contracted with 40 mM KCl and the resulting IEF gels compared. Once again there was no detectable difference, (Fig 9).

IEF of myometrial strips, (Table 4), lacked the sensitivity required to visualize changes in the protein profile of crude myometrial homogenates. The concentration of MLC present in these samples was too small to allow detection of changes in phosphorylation by Coomassie blue staining. For this reason the more sensitive technique utilising ³²P in conjunction with SDS-PAGE was adopted. SDS-PAGE was chosen as this one-dimensional technique allows for limited handling of radioactive material when compared to two-dimensional electrophoresis. It also results in a flat gel ideal for autoradiography. Since myometrial strips appeared to be unresponsive to NG, one of the drugs of choice in this study, rabbit
FIGURE 8:

Scans of myometrium. Orientation as in Figure 5.

A: Myometrium was contracted with 40 mM KCl (10 min) and frozen.

B: Myometrium was frozen relaxed.
A - 40mM KCl

B - Relaxed control
FIGURE 9:

Myometrium were either relaxed in a Ca$^{2+}$ free Tyrodes (A) or contracted in 40 mM KCl (B) as described in the text orientation as in Figure 5.
TABLE 4:

Summary of experiments using IEF to detect changes in phosphorylation.

<table>
<thead>
<tr>
<th>I: Relaxed control vs. KCl (40 mM) 10 min</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>II: KCl (40 mM) 10 min vs. KCl (40 mM) 10 min and ISO (10^-6M, last 1 min)</td>
<td>2</td>
</tr>
<tr>
<td>III: Ca^{2+} free Tyrodes - vs. equilibration in Ca^{2+} free Tyrodes then KCl (40 mM) 5 min</td>
<td>4</td>
</tr>
</tbody>
</table>

No differences in the protein profiles were noted.
aortic strips were used.

2.SDS-PAGE

i) Calcium free study:

In order to show that the technique was working I chose to compare two extreme states. One set of aortic strips (n=6), was labelled with $^{32}$P, and incubated in a Ca$^{++}$ free Krebs buffer containing 5 mM EGTA for 20 min. This ensured that the tissues were completely relaxed. The second set of muscle strips were contracted with 124 mM KCl Krebs buffer in which NaCl had been replaced by KCl in equimolar proportions. This concentration of K$^+$ -depolarised the muscles and produced a maximal sustained contraction. The muscles were frozen after 30 s as this, according to Aksoy et al., (1982), is the point of peak phosphorylation.

Contracted and relaxed muscle strip samples were run on the same gradient gels, to allow easy comparison between the two types of treatments. A typical example of such an autoradiogram is shown in Fig 10. It is obvious from the autoradiogram that there is phosphorylation of a protein, 23kD, in the contracted tissues whereas those strips incubated in a Ca$^{++}$ free Krebs buffer appear to have very little phosphorylation at this point. The molecular weight of the proteins were determined by the method of Weber et al., (1972), by comparing the mobility of the proteins with those of marker proteins run concomitantly on each gel. The autoradiograms were scanned in an attempt to quantitate this change in phosphorylation. Fig 11 shows four such scans. The difference in degree of phosphorylation of the 23kD protein (assumed to be the myosin light chain) is well visualized by the scans. In order to quantitate the results the outline of the scans were cut out and the paper weighed, (DiSalvo et al., 1978). The
FIGURE 10:

Autoradiogram of a SDS polyacrylamide gradient gel.

Tracks shown represent samples:

a) Relaxed in Ca\(^{2+}\)-free (5mM EGTA) Krebs, 20 min
b) Contracted in 124 mM KCl Krebs, 30 s

Preparation of samples, gel and autoradiogram were as described in Methods. Result shown is a typical autoradiogram.
FIGURE 11:

Scans of autoradiograms of SDS-polyacrylamide gradient gels, as described in the text. A and B represent aortic strips contracted in 124 mM KCl and frozen after 30 s. C and D represent aortic strips relaxed in Ca$^{2+}$ free (5 mM EGTA) buffer and frozen after 20 min.

The 23 kD peak was assumed to be myosin light chain (MLC). For rationale see text.
peak at 23kD was expressed as a percentage of the total weight of the scan. The results shown in Table 5 indicate a significant difference between the treatment groups. This was taken as validation of the techniques used.

This 23kD protein can not be definitely identified as MLC from these studies. However, its characteristics are similar to those documented for MLC. The protein is phosphorylated on contraction and dephosphorylated in Ca\(^{2+}\)-free buffer, it also appears as the only protein phosphorylated in that region, 15 - 30kD. For these reasons it was assumed to be MLC.

However there are obvious problems in evaluating the data in this manner. By expressing the change in phosphorylation of the 23kD protein as a percentage of the total phosphorylation the assumption is being made that there are no other increases or decreases in protein phosphorylation. If the scans in Fig 11, between contracted and relaxed muscle strips are compared, there are no other visible changes in phosphorylation levels. It was therefore concluded that although such a method of expressing protein phosphorylation would not produce figures that were an accurate representation of the degree of phosphorylation, significant differences would be apparent and trends in phosphorylation rather than absolute values could be expressed.

Another problem encountered using this technique is the vertical streaking of proteins. This could be caused by several factors: a) It could be sample overload, in which case diluting the sample would be a solution. However, the proteins in the low molecular weight range are already faint and diluting the sample further would possibly dilute out the protein under study. b) Some native proteins form aggregates and may precipitate at the high concentrations reached in the sharply stacked zone in the stacking gel and fail to enter the resolving gel. This phenomenon of
TABLE 5:

Ca\(^{2+}\) Dependency of phosphorylation of 23kD protein in rabbit aorta.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of total phosphorylation in 23kD protein band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+}) free, 5 mM EGTA 20 min</td>
<td>1.7±0.24(6)</td>
</tr>
<tr>
<td>124 mM KCl, 1.26 mM Ca(^{2+}), 30 s</td>
<td>4.6±0.68(6)*</td>
</tr>
</tbody>
</table>

Each value represents the mean±SD of the number of experiments shown in parentheses.

*Significantly different from relaxed phosphorylation values, p<0.05.
streaking occurs when aggregated protein accumulates at the gel surface and then slowly enters the gel during electrophoresis, causing protein streaks running in the direction of migration. To ensure that all insoluble material is removed prior to electrophoresis, samples were centrifuged before application.

ii) Potassium - time course study:

Having established a working protocol to examine MLC phosphorylation levels and considering the controversy in the literature, it seemed pertinent to determine the time point of peak MLC phosphorylation during a K⁺-induced contraction. In addition, before examining the effect of relaxants on phosphorylation it was crucial to determine the degree of phosphorylation at the time of addition of relaxant. A K⁺-time course study was therefore undertaken. The tissues were frozen after contraction with 124 mM K⁺ Krebs buffer at the time points indicated, (Fig. 12). Muscle strips, which were not treated with this depolarising buffer served as relaxed controls.

Tension in the muscle strips reached a maximum after 4 min and this degree of contraction was maintained throughout the duration of the time course. The phosphorylation peaked around 2 min. There then appears to be a trend towards dephosphorylation. The results from the Ca⁺⁺ dependency study are included as the gels were comparable. It is worth noting that the levels of phosphorylation in Ca⁺⁺ free relaxed muscles (1.7% of total phosphorylation of 23kD protein: Table 5) are significantly lower than the relaxed controls (2.4% of total phosphorylation of 23kD protein: Fig 12).
FIGURE 12:

Phosphorylation of MLC during K⁺-induced contraction of rabbit aorta. The solid line represents tension developed by a representative muscle contracted for 12 min. The columns represent the mean MLC phosphate content of muscles (expressed as a % of total phosphorylation) frozen at the indicated times. The error bars represent the standard deviation of the mean for 4 phosphate determinations.

*significantly different from control p<0.05.
**significantly different from 30 s p<0.05.
TENSION (grams) —

MLC PHOSPHORYLATION (% of total)

TIME (min)

0 2 4 6 8 10 12

---

* * *
TABLE 6:

Effect of nitroglycerin (10^{-6}) on K^+-induced contractions.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Maximal Tension</th>
</tr>
</thead>
<tbody>
<tr>
<td>124 mM KCl for 7 min</td>
<td>100% (5)</td>
</tr>
<tr>
<td>124 mM KCl for 7 min,</td>
<td>*30±±28 (5)</td>
</tr>
<tr>
<td>NG(10^{-6}M) for last 2 min</td>
<td></td>
</tr>
</tbody>
</table>

The number of experiments is shown in parentheses.
*Value represents the mean±SD.
FIGURE 13:

Mechanical responses of rabbit aorta to 124 mM KCl and nitroglycerin (10^{-6}M).

Aortic strips were first contracted with 124 mM KCl, and in A, frozen after 7 min. B, nitroglycerin (10^{-6}M) was added for last 2 min and then frozen.
A

KCl
124 mM

NG $10^{-6}$ M

B

KCl
124 mM
**TABLE 7:**

Effect of nitroglycerin (10^{-6}M) on $^{32}$P incorporation of 23kD protein during K$^+$-induced contraction in rabbit aorta.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of total phosphorylation in 23 kD protein band</th>
</tr>
</thead>
<tbody>
<tr>
<td>124 mM KCl for 7 min</td>
<td>3.2±0.66 (5)</td>
</tr>
<tr>
<td>124 mM KCl for 7 min, NG (10^{-6}M) last 2 min</td>
<td>*1.9±1.13 (5)</td>
</tr>
</tbody>
</table>

Each value represents the mean±SD of the number of experiments shown in parentheses.

*Significantly different from contracted phosphorylation values, $p<0.05$. 
FIGURE 14:

Scans of $^{32}$P autoradiograms of SDS polyacrylamide gradient gels, as described in text.

A and B representing aortae contracted in 124 mM KCl, (7 min)
C and D representing aortae contracted in 124 mM KCl, (7 min)
with nitroglycerin ($10^{-6}$M) added in last 2 min.
TABLE 8:

Summary of experiments using SDS-PAGE to detect changes in phosphorylation.

I  Ca\(^{++}\) Free Krebs (5 mM EGTA), 20 min vs. 124 mM KCl, 30 s

II  124 mM KCl, 2, 6 and 12 min vs. relaxed control

III  124 mM KCl, 7 min vs. 124 mM KCl, 7 min plus nitroglycerin (10\(^{-6}\)M) last 2 min
iii) Effect of nitroglycerin (10 M)

Since contraction appears to be correlated with phosphorylation, the relationship between tension and levels of phosphorylation was subsequently examined in muscles relaxed by NG (10⁻⁵ M). The muscle strips were contracted in 124 mM K⁺ Krebs, as previously described, and NG was added directly to the organ baths to produce a final concentration of 10⁻⁵ M. This produced a 70% relaxation, (Table 6). Fig 13 shows typical traces obtained.

The % phosphorylation values of the MLC, for the two treatments are given in Table 7. There is a significant decrease in the % phosphorylation of MLC (3.2% verses 1.9% of total phosphorylation of 23kD protein for 124 mM K⁺ Krebs and nitroglycerin treatment respectively). If the scans of the autoradiograms are compared, (Fig 14), not only is the alteration in the phosphorylation of MLC seen, but there appears to be increased phosphorylation of a protein at approximately 160kD in the muscles treated with NG. In order to quantitate this difference, the sides of the peaks must be extrapolated to the base line. The bases of the peaks from the contracted muscles are wider than their relaxed counterparts. As a result, although the 160kD peak of the NG-treated muscles appears to be elevated, the area under the peak is not significantly different. Due to the other background radioactivity on one-dimensional gels, the values for percent increase in phosphorylation may be somewhat underestimated, (Janis et al, 1981). Table 8 represents a summary of the experiments using SDS-PAGE to detect changes in phosphorylation.
DISCUSSION

The major objective was the establishment of a suitable protocol for determining changes in protein phosphorylation in smooth muscle.

Electrophoresis has become the preferred technique for studying protein phosphorylation in intact muscles. The number of reports in the literature using these techniques is immense, (for reviews see Marston, 1982; Stull et al, 1980; Murphy, 1982; Stull, 1980).

IEF is widely used as the first step in two-dimensional electrophoresis. It is not so common to find it used alone in protein analysis of smooth muscle. Intact smooth muscle homogenates contain many other constituents besides contractile proteins. In these studies, it was estimated that 11% of total wet weight of myometrial tissue and 8% of aortic tissue was protein. This is the total weight of protein in the muscle, of which only a small portion is contractile proteins. Myosin has been estimated by some to be 7% of total protein content of porcine smooth muscle, (Cohen and Murphy, 1978). For this reason many workers have tended to purify their muscle preparations prior to 2-D electrophoresis. Looking at the IEF scans obtained using myometrium, (Fig 7,8,9), it can be seen that the base line at the basic end is elevated, causing differences in phosphorylation to appear less pronounced. From studies using SDS-PAGE, it became apparent that the relative amount of MLC present in these samples was small. So with hindsight it is not surprising that the changes in light chain phosphorylation are not detectable using IEF. For these reasons IEF, when used on its own, was deemed to be an inappropriate method of studying protein phosphorylation in these types of preparations.

Using $^{32}$P overcomes this problem, as autoradiography is much more
sensitive than scanning a stained protein spot. It should be remembered that using SDS-PAGE there is no detectable difference between a phosphorylated or non-phosphorylated protein, as separation is on the basis of size rather than charge. Charge will vary significantly on phosphorylation whereas size will not.

SDS PAGE was more useful particularly when gradient gels were employed. Since there were no other proteins of the same size phosphorylated in that region, (15 - 25kD), the estimation of % phosphorylation of the MLC was easier. SDS-PAGE is limited in its use when proteins of a high molecular weight (150kD and heavier) are studied, (Fig 14). There appears from Fig 10 to be a high level of radioactivity associated with proteins in the higher molecular weight range. The exact nature of this activity cannot be determined from these autoradiograms. It may in part be due to the spreading of the spot on the film due to the long exposure times (7 - 30 days) or it may represent phosphorylated proteins in such low concentrations that they are not adequately resolved by this technique.

Two-dimensional electrophoresis may not necessarily solve this problem. There may still be a large amount of streaking at the top of the gel, except that it would be spread across the width of the gel. Fig 15 illustrates this and detection of phosphorylation in the high molecular weight region would be impossible. Possible solutions to this dilemma are discussed at a later point.

A significant difference in the levels of phosphorylation between K\(^+\) -induced contracted and Ca\(^++\) -free relaxed aortic smooth muscle, was taken as an indication that the methodology was suitable and working. In the studies above, these changes were seen in a 23kD protein. The widely
FIGURE 15:

Autoradiograms of soluble (upper) and particulate (lower) fractions of rat thoracic aorta after two-dimensional gel electrophoresis.

Samples were prepared as in method B, soluble fraction represents supernatant.

Reproduced from Rapoport et al. (1982).
accepted weight of the MLC of smooth muscle is 20kD. The MLC of skeletal muscle is quoted as 18.5kD, (Stull et al., 1980). DiSalvo et al., (1978), estimated the molecular weight of the two types of light chain in bovine aortic actomyosin to be 15kD and 13kD, which corresponds to values for chicken gizzard, (Sobieszek et al., 1977; Aksoy et al., 1976; Gorecka et al., 1976) or platelets, (Chacko et al., 1977) and this reflects the heterogeneity of MLC isolated from different sources, (Mannherz et al., 1976). As is indicated throughout both IEF and SDS-PAGE are sensitive to artifactual variation. Therefore the IEF point and the molecular size of proteins will vary from one laboratory to another. What is important is consistency of results within a laboratory.

i) Effect of Calcium

Perry and his colleagues first described the MLCK/MLCP system (Perry, 1979). However, since MLC phosphorylation had no apparent effect on actin-activated ATPase activity of skeletal muscle, no significance was placed on the phosphorylation of MLC with respect to a possible regulatory role in muscle contraction, (Perry, 1979). The discovery that in smooth muscle MLC phosphorylation preceded an increase in actin-activated myosin ATPase activity led to the hypothesis that calcium plays an important role in smooth muscle contraction by activating the MLCK, which in turns phosphorylates the MLC, which initiates an interaction between actin and myosin, resulting in contraction, (Chacko et al., 1977; Gorecka et al., 1976; Sobieszek A, 1977). This model is based on the assumption that increased ATPase activity is due to increased interaction between a large number of myosin molecules with actin. Relaxation, according to the above hypothesis
would result from Ca\(^{2+}\)-sequestration, MLCK inactivation and subsequent dephosphorylation of MLC by MLCP.

If Ca\(^{2+}\)-induced phosphorylation mediates cross-bridge attachment in living smooth muscle, then active stress should be related to the level of MLC phosphorylation under all conditions. This has been observed many times in the literature.

In the study reported here, unstimulated tissue, in this case relaxed controls, had low levels of phosphorylation (i.e. 2.4% of total phosphorylation observed, Fig 12). In the literature, levels range between 0.1 and 0.2 mol phosphate / mol MLC, (Aksoy et al. 1982; Barron et al, 1979; Butler et al, 1983; de Lanerolle and Stull, 1980; Driska et al, 1981; Gerthoffer and Murphy 1983 a, b; Silver and Stull, 1981). These non zero values in relaxed tissues could be attributed to the presence of tone, (Barron et al, 1979; Driska et al, 1981), errors in phosphorylation determination, (Aksoy et al, 1982; Driska et al, 1981; Manning et al, 1980), or cooperativity between heads of myosin, (Persechini et al, 1981).

During K\(^{+}\)-induced contractions there was a significant increase in MLC phosphorylation, (Table 5). Values in the literature appear as high as 0.7 mol phosphate / mol MLC either before or coincident with tension development, (Aksoy et al, 1982; Barron et al, 1979; Butler et al, 1983; de Lanerolle and Stull, 1980; Gerthoffer and Murphy, 1983 a, b; Janis et al, 1981; Silver and Stull, 1981). Although maximal force is achieved, given values for phosphorylation are submaximal (i.e. less than 1 mol phosphate / mol MLC). This could be caused by the failure of an agonist to completely activate the contractile machinery, or to some threshold or cooperativity phenomenon, (Kerrick et al, 1980).

On removal of Ca\(^{2+}\) from the system using Ca\(^{2+}\) -free Krebs and 5 mM EGTA
there was a further decrease in phosphorylation levels from relaxed controls (1.7% verses 2.4% of total phosphorylation of 23kD protein, Table 5). High basal levels may also be due to spontaneous action potentials within the muscle, which in turn would release Ca\(^{++}\) from the tissue, resulting in a certain degree of phosphorylation. Total removal of Ca\(^{++}\) from the system would eliminate this effect decreasing levels of phosphorylation, (de Lanerolle et al, 1982).

As previously discussed, these crude muscle preparations contain various cells other than smooth muscles cells (for example fibroblasts and endothelial cells and a variety of enzymes such as kinases and phosphatases, some of which are also involved in phosphorylation reactions). Fig 10 illustrates this point. Superimposed on the elevated level of phosphorylation, definite bands can be seen, with apparent molecular weights of 28, 37, 42, 78, 94, 137, 160 kD. The degree of phosphorylation of these bands does not seem to be altered by any of the treatments in this study, with the exception of the 160 kD protein, which appears to have increased phosphorylation with NG, (Fig 14).

Aside from the two proteins which have already been discussed (i.e. MLC and the 160kD protein), the identity of the other phosphorylated proteins, which appear on the gels, is as yet unknown. It should be stressed again that this is a crude muscle homogenate and so there are many proteins and enzymes present and these phosphorylations may have no bearing on contraction.

ii) Potassium - time course study.

Before the effect of relaxants on phosphorylation levels can be determined it is important to establish the time course of MLC
phosphorylation during a K\(^+\)-induced contraction and to determine at what time point peak phosphorylation levels are attained. As was alluded to in the introduction, there is some controversy as to the time course of phosphorylation with respect to contraction. Fig 16 (a+b) summarises the two concepts presented in the literature. Fig 16a is the time course obtained by Aksoy et al. (1982). This study was conducted in swine carotid arteries using K\(^+\) as a stimulant. Phosphorylation was determined by 2D electrophoresis and scanning of the light chain spots. From this study the authors concluded that MLC phosphorylation levels rapidly increased to peak values with stimulation and then declined significantly, although stress continued to rise to a maximum, (Aksoy et al, 1982).

In Fig. 16b, the study was conducted on canine tracheal muscle using methacholine as the stimulant. Phosphorylation was determined by IEF of purified myosin. The findings of this study were that the level of MLC phosphorylation rose with tension development to a maximum, where it remained through the study.

There were a few differences between these two studies worth comment; a) Vascular and non-vascular smooth muscles are being compared. Vascular smooth muscle is known to have a high degree of tone, (Aksoy et al, 1982) Its physiological role is obviously different and thus different characteristics would be important. A method by which energy could be conserved during contraction (i.e. via latch bridges) would be advantageous in the case of vascular smooth muscle. b) The two preparations used are of different thickness and this may alter the penetration of the agonist into the tissue and it's effect with respect to time. c) Different stimulants were used, K\(^+\) in the case of the former study and methacholine in the latter. Although they have at least one mode of action in common, that is
FIGURE 16:

A. Time course of MLC phosphorylation stress development, and shortening velocity in K\(^+\)-stimulated carotid media tissues. Each phosphorylation measurement (n=42) was obtained on single tissue frozen at indicated time during isometric contraction (●, standard freezing protocol by immersion in acetone-dry ice slurry; *, quick frozen using metal clamps precooled in liquid nitrogen; ©, immersion freezing after step shortening. Mechanical data are reproduced from Dillan et al., 1981.

Reproduced from Aksoy et al. (1982).

B. Myosin phosphorylation during methacholine induced contraction of tracheal smooth muscle. The solid line represents tension developed by a representative muscle contracted for 15 min. The columns represent the mean myosin phosphate content of muscles frozen at the indicated times. The error bars represent the standard error of the mean for five to seven phosphate determinations.

Reproduced from de Lanerolle et al. (1980).
MOLES PHOSPHATE PER MOLE MYOSIN

TENSION (grams)

TIME (seconds)

MOLES PHOSPHATE PER MOLE MYOSIN

Velocity (10^-5 N/m)

Force/Area (10^-5 N/m)

Moles P/Mole LC 20

Sec

Min

60
40
20
0
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
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62
64
66
68
70
72
74
76
78
80
82
84
86
88
90
92
94
96
98
100

10^-4 M

m g KSCN

8 M

Meth.

TENSION (grams)
they cause depolarisation of smooth muscle, methacholine may be exerting other effects causing the differences noted.

The results presented in this study fall between the two camps. Vascular muscle was used, as was K+ as a stimulant, in an attempt to reproduce Murphy and colleagues' findings, (Aksoy et al, 1982). However such a marked decrease was not seen, (Fig 12). There appeared to be a decreasing trend but this was not significant at the levels tested and without later time points, no firm conclusion can be made. A main disadvantage of the method of quantitating MLC phosphorylation as used in this study, is that results obtained cannot be compared with results quoted in the literature.

iii) Nitroglycerin - induced relaxation

The observed decrease in levels of phosphorylation of MLC on relaxation of K+-induced contractions with NG (10^-6 M) were predicted by the original hypothesis that MLC phosphorylation initiates an interaction between actin and myosin resulting in contraction. Whether the rate of MLC dephosphorylation is altered by NG is worth investigating as it cannot be determined from this experiment. According to Driska et al, (1981), if swine carotid arterial strips are contracted with K+ for 2 min and the stimulus washed out, the levels of phosphorylation have reached basal values before force has declined by 50%. In this study levels of phosphorylation decreased to basal levels with NG even though there was a degree of tension left in the muscle. However it should also be noted that the values for the K+-contracted muscle strips, which acted as a control, were lower than those expected from the time course. The reason for this is not clear. It can be concluded that both stimulus washout and NG-induced
relaxation cause dephosphorylation.

It is difficult to speculate whether a decrease in levels of phosphorylation seen with NG treatment is necessarily causing relaxation. The fact that there is substantial proof that muscles can maintain tone, with reduced levels of phosphorylation, complicates the data interpretation. Murphy and co-workers have analysed the relaxation of carotid arteries following agonist (110 mM K⁺) washout. It was found that the kinetics of relaxation could be described by a dual exponential decay. The initial phase (about 2 min) was rapid and depended on the length of time of agonist stimulation. It also correlated with MLC dephosphorylation and isotonic shortening velocities. It was concluded that this phase was associated with the phosphorylated rapid-cycling cross-bridges, which appeared to dephosphorylate with time during a sustained K⁺-induced contraction. Hence it was dependent on the length of time of agonist stimulation.

The second phase was much slower (estimated at 45 min). This phase was sensitive to Ca²⁺ concentration. Decreases in Ca²⁺ concentration (0 mM Ca²⁺, 0.1 mM EGTA) greatly reduce the duration of this phase. Increasing Ca²⁺ concentrations to 5 mM results in prolongation. It was concluded that this was a second regulatory mechanism present in smooth muscle, referred to as latch bridges which are dephosphorylated cross bridges maintaining stress, at a greatly reduced energy level, (Gerthoffer and Murphy, 1983b).

A recent study by Gerthoffer et al (in press) looked at the effect of SNP on this second phase of relaxation. SNP is thought to cause relaxation in a manner similar to NG. These two vasodilators activate guanylate cyclase directly via the reactive intermediate NO, (Gruetter et al, 1979, 1980, 1981). There appears to be a close correlation between the degree of
smooth muscle relaxation and the extent of cGMP formation, (Katsuki and Murad, 1977, Axelsson et al. 1979, Kukovetz et al. 1979). The other conclusion drawn from the study by Gerthoffer and coworkers is that SNP will enhance the second phase of relaxation in carotid arteries without decreasing the already low levels of phosphorylation (i.e. this vasodilator exerts its effect on the Ca^{2+}-dependent stress maintained by latch bridges rather than on the MLCK/MLCP system).

The other interesting feature noted in the NG study presented here was the apparent increase in phosphorylation of a 160kD protein in the muscles treated with NG. This increase was not significant, by the methods used, though a possible explanation for this has previously been discussed.

NG causes an increase in cGMP levels and this protein phosphorylation could result from altered activities of a protein kinase or a phosphoprotein phosphatase or both. What this alteration is, what the 160kD protein is and whether this phosphorylation has pharmacological bearing are questions which merit further study.

The nature of the 160kD protein is unknown. These preparations are crude muscle homogenates. One way of determining whether this is a contractile protein would be to purify the homogenate and run an SDS-PAGE of the myofibrils only. One of the problems in dealing with a protein of this size is that proteins do not appear as discrete bands due to vertical streaking. IEF may be a method of solving this if the phosphorylation changes can be easily spotted. Due to the abundance of other cellular material, smooth muscle homogenates result in gels with varying baseline levels of staining due to incomplete focusing of certain proteins.

The phosphorylation might be cGMP-dependent but that would have to be firmly established by treating aortic strips with other drugs known to
increase cGMP levels (e.g. SNP). Another way to test this would be with in vitro studies adding cGMP in a fashion similar to in vitro studies on the cAMP-dependent protein kinase system. Is this effect restricted to aortic smooth muscle or is it seen in other vascular and non-vascular muscle? It would be a convenient answer to the controversial problem of why SNP produces large increases in cGMP levels in K\textsuperscript{+}-contracted strips of vas deferens without causing relaxation. (Diamond, 1978.) Could it be that this 160kD protein is involved in relaxation and is not phosphorylated by SNP in rat vas deferens? Comparative studies using vascular and non-vascular smooth muscle should provide valuable information regarding these possibilities. If phosphorylation occurs in both types of muscle this would indicate that either the protein phosphorylation is not responsible for relaxation or that some other factor is missing in non-responsive tissues. One could speculate endlessly, but only further investigations will give the answers.

A similar study with SNP and rat thoracic aorta was conducted by Rapoport et al., (1982). This group found a concentration-dependent increase in the phosphorylation of nine proteins and a decrease in the phosphorylation of two proteins, with SNP. This effect appeared to be mimicked by cGMP analogues. However the 2-D gels published in this study do not visually demonstrate this finding and these changes were noted by using a video camera scanning system with an analog-to-digital converter and a microcomputer. Neither were any values for phosphorylation levels quoted.

All of the proteins in the above study are in the molecular weight range 21-49kD and there was no report of a change in the higher molecular weight proteins. The phosphorylations observed may in fact be due to activation of a cGMP-dependent protein kinase by SNP and NG, but may have
nothing to do with the mechanisms of muscle relaxation. Conversely, proteins involved in the regulation of muscle relaxation may be phosphorylated in NG-relaxed muscles by kinases other than those which are cGMP-dependent.
SUMMARY AND CONCLUSION

1) Although IEF is used on its own as a method of protein separation in skeletal muscle studies, this technique was deemed unsuitable for the analysis of crude homogenates of smooth muscle.

2) A working technique was established in the laboratory in which changes in MLC phosphorylation could be examined.

3) The degree of MLC phosphorylation with respect to time during K⁺-induced contractions appears to correlate with tension. However, further time points are needed to determine if the decreasing trend in phosphorylation is significant.

4) NG induces relaxation and a dephosphorylation of the MLC. There also appears to be an increase in the phosphorylation levels of a 160kD protein with NG treatment. This apparent increase merits further study and may lead to an explanation of possible anomalies found in the literature, with respect to vasodilators of this type and their methods of action.
BIBLIOGRAPHY


