Biochem. J. (1982) **204**, 257–264
Printed in Great Britain

The effects of calcium on protein turnover in skeletal muscles of the rat

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(*Received 23 October 1981/Accepted 20 January 1982*)

Several experimental procedures were used to increase the intracellular concentration of Ca^{2+} and determine its effects on protein turnover in isolated extensor digitorum longus and soleus muscles. These methods included the use of ionophore A23187, caffeine, dibucaine, thymol and procaine, all agents known to induce the release of calcium by acting either on the sarcolemma and/or on the sarcoplasmic reticulum. Another approach involved varying the external concentration of Ca^{2+} in the media in which the muscles were incubated. The changes in muscle Ca^{2+} concentrations after exposure to the various calcium-releasing agents were in keeping with accepted modes of action of these agents on muscle membranes. The findings suggest that increasing the sarcoplasmic concentration of Ca^{2+} inhibits protein synthesis and enhances protein breakdown. These catabolic effects of Ca^{2+} are compared with the changes induced in muscle protein turnover after exposure to insulin or cyclic nucleotides, and in myopathic muscle and situations of work overload. Attention is also drawn to some of the difficulties involved in definitively implicating Ca^{2+} as a factor involved in the normal regulation of protein turnover.

Calcium is known to be important in regulating many cellular functions. For example, it participates as a cofactor in the release of neurotransmitter substances and of histamine from mast cells, and in the secretion of the products of exocrine and endocrine glands. This bivalent cation may also act as a second messenger in certain hormone actions, e.g. in the regulation of various steps of carbohydrate metabolism by insulin (Clausen & Martin, 1977; Schudt & Pette, 1978). In addition, Ca^{2+} is an essential requirement for the fusion and metabolic maturation of myoblasts (Yaffe, 1969; Schudt & Pette, 1978) and in the normal coupling of excitation and contraction in striated muscle (Ashley & Ridgeway, 1970; Ebashi, 1972; Allen & Blinks, 1978).

In addition to its role in excitation–contraction coupling, the changing free concentration of Ca^{2+} in the sarcoplasm of muscle may also serve as an intermediate, linking contractile activity and its known effects on muscle protein turnover (Goldspink, 1980). Deficiencies in this normal regulation of free Ca^{2+} may explain some of the abnormalities seen in myopathic muscle. For example, in the hereditary muscular dystrophies, defective membrane structures and functions may be responsible for the observed deposition of Ca^{2+} within some fibres (Engel, 1977; Emery & Burt, 1980). It is also known that the sarcoplasmic reticulum of dystrophic muscle is unable to sequester Ca^{2+} as efficiently as it does in the normal tissue (Samaha & Gergely, 1969; Takagi *et al.* 1973). Such observations have led to the suggestion that an inability to regulate intracellular concentrations of Ca^{2+} correctly may explain the stimulation of both protein synthesis and protein breakdown in dystrophic muscle (Simon *et al.*, 1962; Srivastava, 1969; Ionasescu *et al.*, 1971). Similarly, the increases in protein turnover found in hearts subjected to volume or pressure overloads (Morkin *et al.*, 1972) and in skeletal muscles stretched by immobilization (Goldspink, 1977) or by the attachment of external weights (Laurent *et al.*, 1978) could conceivably be related to induced changes in the ionic permeabilities of muscle membranes. Indeed, some more-recent studies (Kameyama & Etlinger, 1979; Etlinger *et al.*, 1980; Sugden, 1980) have indicated that increasing intracellular Ca^{2+} concentrations in muscle can lead to similar changes in protein turnover to those described above for functionally overloaded or dystrophic muscle.

Such possible roles of Ca^{2+} in both normal and abnormal tissues have prompted much current interest in the study of this cation. The present

Abbreviation used: EDL, extensor digitorum longus.

0306-3283/82/040257-08$01.50/1

investigation was undertaken to re-evaluate some of these possible roles of Ca^{2+}, and in particular to determine how this bivalent cation might mediate changes in the rates of protein synthesis and protein breakdown in muscle. Attention is also drawn to the difficulties in studying the precise role of Ca^{2+} in any system, and in particular in skeletal muscle. To minimize possible technical artefacts or non-specific actions of drugs associated with the release of Ca^{2+}, we have used several experimental approaches, each designed to alter the sarcoplasmic concentration of Ca^{2+} in isolated muscle preparations.

Experimental

All experiments involved the use of young male rats (CD strain; approx. 50g) obtained from Charles River U.K. Ltd., Manston, Kent, U.K. Animals were killed by cervical dislocation and the extensor digitorum longus (EDL) or soleus muscles were rapidly dissected out, weighed and immersed in the appropriate incubation medium for 2h at 37°C. Average rates of protein synthesis and protein breakdown were measured in these intact isolated muscles by the slightly modified (Goldspink, 1978) method of Fulks *et al.* (1975). These measurements *in vitro* were made in an oxygenated (O_2/CO_2, 19:1) medium, consisting of Krebs–Ringer bicarbonate buffer (DeLuca & Cohen, 1964) containing 10mM-glucose, 5 times plasma concentrations of all amino acids (Mallette *et al.*, 1969) and 0.01 unit of insulin/ml. For measurements of protein synthesis, 0.15μCi of L-[U-^{14}C]tyrosine (sp. radioactivity 483mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K)/ml was added to the basic medium and the incorporation of the amino acid tyrosine into muscle proteins was determined after a 2h incubation. The total number of moles of tyrosine incorporated was calculated by dividing the measured [^{14}C]tyrosine covalently bound in protein by the specific radioactivity of the intracellular tyrosine pool (Goldspink, 1978).

Protein degradation was determined by measuring the release of tyrosine (Waalkes & Udenfriend, 1957) from muscle proteins into intracellular amino acid pools and into the surrounding medium after a 2h incubation. For these measurements of breakdown, muscles were incubated in a medium identical with that for synthesis except for the omission of tyrosine in order to increase the sensitivity of the system for measuring the released tyrosine, and the inclusion of cycloheximide (50μM) to block protein synthesis, thereby preventing reutilization of the released tyrosine.

The Ca^{2+}-releasing agents ionophore A23187, procaine hydrochloride, dibucaine, caffeine and thymol (all obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.) were added to the appropriate media in the doses shown below. In experiments where the external calcium concentrations were manipulated, various concentrations of $CaCl_2$ (0–5mM) were added to the incubation media, with EGTA (2mM) being added to those media containing no $CaCl_2$.

Results

Several experiments were undertaken to study the effects of modifying the sarcoplasmic concentration of Ca^{2+} on protein turnover in small isolated muscles. The intracellular Ca^{2+} concentrations were changed either by the use of the specific bivalent ionophore, A23187, or by exposing muscles to another group of drugs which increase the Ca^{2+} permeability of the sarcolemma and/or the sarcoplasmic reticulum, or simply by altering the concentration of Ca^{2+} within the incubation medium.

Since the changes induced in the EDL and soleus muscles were very similar, only part of the results obtained for the soleus muscle are presented below.

Effects of bivalent cation ionophore A23187

Ionophore A23187 is a monocarboxylic acid antibiotic which increases the movement of calcium across membranes. Its relative specificity has led to its use in the study of many Ca^{2+}-dependent cellular functions. Initially the influence of various concentrations of ionophore A23187 on protein turnover in the EDL (Fig. 1) and soleus (results not shown) muscles was studied in the presence of normal levels of external Ca^{2+} (2.5mM). All concentrations of ionophore A23187 (1–10^4ng/ml) significantly ($P<0.025$) inhibited protein synthesis, with decreases of 38–67% in the synthetic rate occurring between the lowest and highest concentrations of ionophore respectively. In marked contrast, none of the ionophore concentrations used led to any significant changes in protein breakdown (Fig. 1).

It has previously been reported (Kameyama & Etlinger, 1979; Sugden, 1980) that the effects of ionophore A23187 *in vitro* on protein turnover are only observed in the presence of external Ca^{2+}. However, as shown in Table 1, we consistently found that ionophore A23187 significantly inhibited protein synthesis in both the EDL and soleus muscles, in either the presence or the absence of external Ca^{2+}. Nonetheless, the inhibition in both muscles in response to ionophore A23187 was always greater in the presence of external Ca^{2+}, thus indicating a partial requirement for external Ca^{2+}. The observable effect of ionophore A23187 in the absence of external Ca^{2+} may be explained by the direct action of the ionophore on the permeability of the sarcoplasmic reticulum, as well as on the sarcolemma (Statham *et al.*, 1976; Rasmussen &

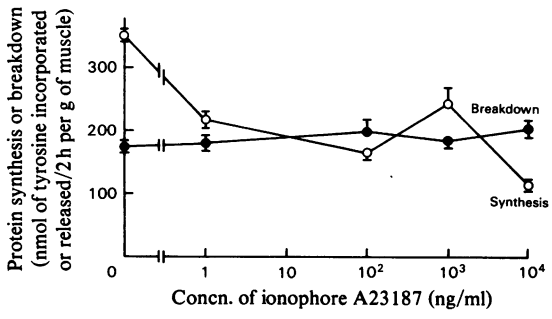

Fig. 1. *Effects of various concentrations of ionophore A23187 on rates of protein synthesis* (○) *and breakdown* (●) *in the EDL muscle in vitro*

Average rates of protein synthesis and protein breakdown were measured in the EDL muscle (see the Experimental section) after 2h exposure to various concentrations (1–10^4ng/ml) of ionophore A23187. The ionophore was initially dissolved in ethanol and subsequently added to the incubation media such that the final ethanol concentration was 0.5%. All muscles incubated in the presence of the different concentrations of ionophore A23187 showed statistically significant decreases ($P < 0.025$) in protein synthesis, compared with control muscles incubated with 0.5% ethanol but without ionophore A23187. Protein breakdown was not significantly altered by the presence of ionophore A23187 at any of the concentrations studied. Statistical evaluations were made by Student's *t* test. Each value represents the mean ± S.E.M. of measurements on eight separate EDL muscles. Soleus muscles were also found to behave similarly on exposure to ionophore A23187 (results not shown).

Goodman, 1977). Hence, in the absence of external Ca^{2+}, the sarcoplasmic concentration of Ca^{2+} may still be increased, but as a result of the release of endogenously stored Ca^{2+}. The action of ionophore A23187 on the sarcolemma system was supported by the increased Ca^{2+} content of the whole muscle when normal extracellular concentrations of Ca^{2+} were present (see below; Fig. 3). The ionophore's action on the sarcoplasmic reticulum is less readily demonstrated. Predictably, an increase in the Ca^{2+} concentration of the sarcoplasma owing to ionophore A23187 action on this particular membrane system would not be reflected as an increase in the whole muscle's Ca^{2+} content (Fig. 3; ionophore-A23187 action in the absence of external calcium).

As stated above, protein breakdown was not significantly affected in either muscle by the ionophore (Table 1), regardless of the concentration used (Fig. 1), or the presence or absence of external Ca^{2+} (Table 1).

Effects of dibucaine, procaine, thymol and caffeine

Local anaesthetics such as dibucaine and procaine are thought to cause the displacement of Ca^{2+} from fragmented preparations of sarcolemma (Thorpe & Seeman, 1971). The other Ca^{2+}-releasing agents used in the present study were the stimulating alkaloid caffeine and the antibacterial phenol thymol. These latter agents have previously been shown to be effective in releasing Ca^{2+} from the sarcoplasmic reticulum of amphibian (Ogawa, 1970) muscle. The overall and relative effects of these Ca^{2+}-releasing agents on protein turnover was therefore studied in our isolated intact muscle preparations.

All four Ca^{2+}-releasing drugs had a marked effect on the muscles, significantly inhibiting the synthesis of proteins in the EDL (Table 2) and soleus (results not shown). The same drugs were also extremely effective in enhancing protein breakdown, except for caffeine, which was without significant effect (Table 2). Although not equally potent (see below), each drug influenced protein turnover in a dose-dependent manner, with the effects becoming more pronounced with increasing concentrations (Table 2). At concentrations of 1 and 10mM, dibucaine completely suppressed protein synthesis while greatly enhancing protein degradation, thereby throwing the muscle into a severe negative nitrogen balance. By comparison, this local anaesthetic was dose-for-dose more potent than its analogue, procaine. Thymol was similarly effective at relatively low concentrations, whereas caffeine at concentrations (e.g. 10mM) comparable with those used in some physiological studies on ion fluxes across the sarcolemma (Bittar *et al.*, 1974) completely arrested protein synthesis (Table 2). Since three of the four drugs used were consistently effective in both inhibiting protein synthesis and stimulating protein breakdown, some support for a common mode of action (i.e. Ca^{2+} releasing) clearly exists.

Without exception, all of the procedures used consistently and profoundly inhibited the rate of protein synthesis (Fig. 1; Tables 1 and 2). At the same time no significant changes were found in either the size or the specific radioactivity of the intracellular tyrosine pool. However, since the induced effects on protein breakdown were less clear-cut, we clarified the situation by means of a further experimental procedure.

Effects of varying the external concentration of Ca^{2+} on protein breakdown

EDL muscles were incubated for 2h in media containing Ca^{2+} concentrations ranging from 0 to 5mM. Decreasing the external Ca^{2+} concentration below that normally present in Krebs–Ringer bicarbonate medium and plasma (2.5mM) suppressed

Table 1. *Effect of ionophore A23187 of the protein turnover of the EDL and soleus muscles*
Muscles were incubated in normal media (see the Experimental section) and exposed to ionophore A23187 (10^4ng/ml), (A) in the presence (2.5mM) or (B) in the complete absence of external calcium (+2mM-EGTA) for 2h. Each value presented is the mean ± S.E.M. of determinations made on eight separate EDL or soleus muscles. The above data are typical of a number of experiments undertaken, the precise number of separate experiments being indicated as the number in parentheses. $*P < 0.001$ and $**P < 0.025$, by Student's *t* test (NS, not significant).

	Protein synthesis (nmol of tyrosine incorporated/ 2h per g of muscle)	Protein breakdown (nmol of tyrosine released/ 2h per g of muscle)
EDL		
(A) $+Ca^{2+}$ (2.5mM)		
Control	245 ± 20	265 ± 14
+A23187	128 ± 10	206 ± 17
% change	−48* (4)	−17 NS (4)
(B) $-Ca^{2+}$ + EGTA (2mM)		
Control	209 ± 20	152 ± 11
+A23187	151 ± 11	155 ± 15
% change	−28* (3)	+2 NS (3)
Soleus		
(A) $+Ca^{2+}$ (2.5mM)		
Control	318 ± 18	471 ± 19
+A23187	149 ± 7	519 ± 14
% change	−53* (3)	+10 NS (2)
(B) $-Ca^{2+}$ + EGTA (2mM)		
Control	237 ± 10	413 ± 23
+A23187	185 ± 16	420 ± 28
% change	−22** (3)	+2 NS (2)

the rate of protein breakdown (Fig. 2). Raising Ca^{2+} concentrations above normal was not easily achieved, and required frequent gassing of the media to prevent Ca^{2+} precipitating out. However, at Ca^{2+} concentrations above 2.5mM, protein breakdown was significantly increased (Fig. 2). Hence, taken alongside the effects of dibucaine, procaine and thymol (Table 2) (but not A23187 and caffeine; Tables 1 and 2), increasing cytosolic Ca^{2+} probably enhances the rate of protein degradation, in addition to inhibiting protein synthesis (Fig. 1; Tables 1 and 2).

Changes in the Ca^{2+} content of the EDL

The ability of the various methods to increase the total Ca^{2+} content within isolated EDL muscles was investigated. From the outset it must be emphasized that these measurements only point to the occurrence of net movements of Ca^{2+} across the sarcolemma. They do not indicate the relative concentrations of Ca^{2+} as distributed between the cytosol, sarcoplasmic reticulum and mitochondria at any given time or in response to any treatments. Nor in fact do they define the physical state of the calcium, i.e. ionized or complexed to cellular constituents such as the parvalbumins, other proteins or phosphates.

As the external Ca^{2+} concentrations were raised or lowered from the normal (2.5mM), so also, in like manner, were the total Ca^{2+} concentrations within the muscle (Fig. 3). Similarly, with the addition of ionophore A23187 (10^4ng/ml in presence of external Ca^{2+}) or dibucaine (1mM) to the normal media, the muscle's Ca^{2+} content increased by 54% and 63% (both $P < 0.001$) respectively (Fig. 3). These changes were again in keeping with the known actions of these substances in increasing the permeability of the sarcolemma to external Ca^{2+}. In contrast, neither caffeine nor thymol at 1 or 10mM, or ionophore A23187 in the absence of external Ca^{2+}, caused any significant changes in the muscle's Ca^{2+} content (Fig. 3), despite inducing changes in protein turnover (Tables 1 and 2). However, under these conditions no change in the total Ca^{2+} content was expected. These particular agents are believed to act on the membranes of the sarcoplasmic reticulum and may thereby effectively increase the concentration of cytosolic Ca^{2+} purely at the expense of endogenous stores; that is, a simple redistribution of the compartmentalized internal Ca^{2+} without affecting the total content within the whole tissue. Hence all of these observations, both positive and negative, are in line with the known actions of these agents on the various membrane systems of skeletal muscle.

Effects of cyclic AMP, cyclic GMP and insulin on protein turnover

Sarcoplasmic influxes of Ca^{2+} are known to modify the intracellular concentrations of cyclic AMP and cyclic GMP. Also, by interaction with

Table 2. *Effect of Ca^{2+}-releasing drugs on protein turnover of the EDL*

EDL muscles were incubated for 2h in the absence (controls) or presence of the appropriate drugs at the final concentrations indicated below. Since several experiments were conducted on different days and the basal rates varied slightly each day, the results are expressed as percentage differences between the mean value for each drug-treated group of tissues and the mean for their respective controls. Each group of tissues consisted of a minimum of six EDL muscles. Near-identical results were obtained when soleus muscles were similarly exposed to these drugs. The data below on the EDL are therefore representative of the type of changes induced in the soleus muscle also. $*P < 0.001$.

		Change owing to presence of drug (%)	
Drug	Concn. (mM)	Protein synthesis	Protein breakdown
Dibucaine	0.1	−21*	+12*
	1.0	−96*	+110*
	10.0	−98*	
Procaine	0.1	−24*	+17*
	0.5		+26*
	1.0	−40*	
Thymol	0.05	−51*	
	0.1		+16*
	0.5	−89*	+58*
	1.0		+76*
	10.0		+140*
Caffeine	0.1	−16*	+1
	1.0	−47*	−2
	10.0	−97*	+9

these cyclic nucleotides Ca^{2+} can participate in various feedback loops, which may modify the subsequent rate of entry, or sequestration, of Ca^{2+} across muscle membranes (Rasmussen & Goodman, 1977). In addition, caffeine, as well as enhancing the movement of Ca^{2+} across the sarcoplasmic reticulum, increases intracellular cyclic AMP by inhibiting the phosphodiesterase enzyme. Hence, in an attempt to determine precisely what effects cyclic AMP and cyclic GMP have on protein turnover, we added the dibutyryl forms of these nucleotides to the appropriate incubation media. Both the protein-synthetic and degradative rates of the EDL muscle (soleus was not studied) were increased after a 2h incubation in the presence of cyclic AMP or cyclic GMP (Table 3). These changes, and in particular those relating to protein synthesis, are in the opposite direction to those consistently induced by the Ca^{2+}-releasing procedures (Fig. 1; Tables 1 and 2). Hence it is improbable that changes in the concentrations of

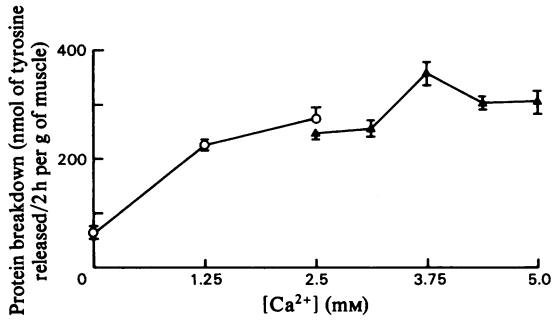

Fig. 2. *Effects of varying the external Ca^{2+} concentration on protein breakdown in the EDL muscle*

In two different experiments (○ and ▲) EDL muscles were incubated in normal media (see the Experimental section), except for the concentrations of $CaCl_2$ as indicated above. In the total absence of calcium, EGTA (2mM) was added to the media; this chelating agent does not of itself influence the rate of breakdown (results not presented). Each value represents the mean ± S.E.M. of measurements on eight separate muscles.

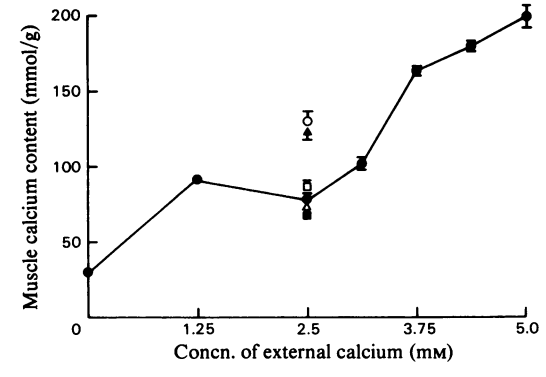

Fig. 3. *Calcium content of the EDL muscle in relation to changes in the external concentration of calcium*

Isolated EDL muscles were incubated for 2h in 1mM-dibucaine (○), ionophore A23187 (10^4ng/ml) in the presence (▲) and absence (△) of 2.5mM external calcium, 1mM- or 10mM-caffeine (□), 1mM- or 10mM-thymol (■) or various concentrations of external calcium (●). The calcium content was then measured with a Pye Unicam atomic-absorption spectrophotometer (model SP.191) after prior homogenization, evaporation to dryness and overnight extraction in a solution of La_2O_3/HNO_3. Each value represents the mean ± S.E.M. for six EDL muscles. At all external calcium concentrations except 1.25mM, changes in the calcium content of the muscles were significantly different ($P < 0.025$ or better) from the value at 2.5mM-calcium.

Table 3. *Effects of cyclic AMP, cyclic GMP and insulin on protein turnover in the EDL muscle*
EDL muscles were incubated for 2h in the appropriate basic media (see the Experimental section) with the subsequent addition of the dibutyryl form of cyclic AMP or cyclic GMP, or insulin. Since insulin binds rapidly (within 2min) and extensively (95%) to glass vessels, the effective concentration of insulin with time was initially determined throughout the incubation period by using a radioimmunoassay (Radiochemicals Kit: The Radiochemical Centre). Subsequently, insulin was initially added to sufficient excess to allow for these complications and to provide the effective number of insulin units shown below. The soleus muscles were not studied in these experiments. $^*P < 0.01$.

	Protein synthesis		Protein breakdown	
	(nmol of tyrosine incorporated/ 2h per g of muscle)	(% change)	(nmol of tyrosine released/ 2h per g of muscle)	(% change)
Control	320 ± 22		293 ± 7	
+1 mM-cyclic AMP	419 ± 26	+31*	442 ± 17	+51*
+0.1 mM-cyclic GMP	477 ± 25	+49*	388 ± 10	+32*
+1 mM-cyclic GMP	387 ± 20	+21*		
Control (without insulin)	161 ± 10		372 ± 21	
+0.1 munit of insulin	239 ± 10	+48*	260 ± 20	−30*
+1 munit of insulin	267 ± 15	+66*	204 ± 15	−45*

intracellular cyclic nucleotides will have mediated the effects that we are attributing to Ca^{2+}.

Since Ca^{2+} has been cited as a second messenger in some actions of insulin (Schudt & Pette, 1978), we also studied the effects of physiological concentrations of insulin on protein turnover in these isolated muscle preparations. In agreement with several previous studies *in vivo* and *in vitro* (Fulks *et al.*, 1975; Rannels *et al.*, 1977; Frayn & Maycock, 1979), we found that insulin both enhanced protein synthesis and inhibited protein breakdown, thus restoring a positive nitrogen balance to the muscle (Table 3). Clearly these anabolic changes induced by insulin do not correlate with the catabolic effects produced by the Ca^{2+}-releasing methods described above. It therefore seems unlikely that insulin's effects on protein metabolism are mediated purely by modifying the sarcoplasmic concentrations of Ca^{2+}.

Discussion

The weight of our experimental evidence suggests that increasing the concentration of sarcoplasmic Ca^{2+} probably inhibits protein synthesis and enhances protein breakdown, thereby inducing a catabolic state. Although Etlinger *et al.* (1980) reached a similar conclusion, important differences exist between these two studies (see below). The increase in protein degradation in association with increasing intracellular concentrations of Ca^{2+} (Table 2; Fig. 2) is in general agreement with earlier studies (Kameyama & Etlinger, 1979; Etlinger *et al.*, 1980; Sugden, 1980). Since a wide variety of experimental approaches have now been used in all of these studies, the possibility that some coupling agent(s) other than Ca^{2+} may be responsible for these observations becomes less likely and the direct involvement of Ca^{2+} increasingly plausible. A Ca^{2+}-induced increase in protein breakdown is consistent with the structural changes observed in muscle (Statham *et al.*, 1976) and may be linked to a Ca^{2+}-stimulated release of lysosomal enzymes (Rasmussen & Goodman, 1977) and/or an activation of Ca^{2+}-sensitive lysosomal and sarcoplasmic proteinases (Pennington, 1977). In physiological terms such mechanisms are still speculative, since the release of lysosomal enzymes appears to be an indiscriminate way of degrading cellular proteins and the Ca^{2+} requirements for activation of such proteinases are usually well in excess of the Ca^{2+} concentrations normally present within the sarcoplasm.

The rates of protein synthesis measured in the isolated EDL and soleus muscles were always inhibited by the methods used to increase the sarcoplasmic concentrations of Ca^{2+} (Tables 1 and 2; Fig. 1). For reasons as yet unknown, these findings conflict with those of Kameyama & Etlinger (1979) and Etlinger *et al.* (1980), who found an enhancement of protein synthesis in muscles exposed to ionophore A23187. However, these same authors did indicate that occasionally ionophore A23187 inhibited protein synthesis in some soleus muscle preparations (Kameyama & Etlinger, 1979). In our hands, this ionophore (and all other agents; Table 2) always suppressed the synthetic rate, regardless of the muscle type and prior (1h), or simultaneous, exposure to ionophore A23187 when physically measuring the rate of synthesis. In these respects our findings are in good agreement with several other investigations in which it was concluded that protein synthesis was suppressed in a variety of tissues and cell types upon increasing their intracellular concentrations of Ca^{2+} (Bottenstein &

de Vellis, 1976; Kanagasuntheram & Lim, 1978; Nakamura & Hall, 1978). The rapid (within 2h) and marked suppression of protein synthesis may suggest an inhibition of the initiation of translation, possibly through inactivation of initiation factors and/or a depletion of energy supplies. Indeed, Sugden (1980) has shown that one of the effects of ionophore A23187 was to decrease the intracellular concentration of ATP, and it is well known that translation is an energy-sensitive process.

Despite the evidence presented from this varied experimental approach, we consider that caution should still be expressed in asserting that Ca^{2+} was directly responsible for inducing the changes in protein turnover. No single method used for increasing intracellular Ca^{2+} concentration is specific to that end alone. For example, caffeine also influences intracellular cyclic AMP concentrations and Na^+ fluxes across membranes (Bittar *et al.*, 1974), the local anaesthetics decrease membrane permeabilities to K^+, and ionophore A23187 may increase membrane permeabilities to other important bivalent cations such as Mg^{2+} (Reed & Lardy, 1972) and alter the resting membrane potential of muscles (Statham *et al.*, 1976). In addition, Ca^{2+} itself exerts a multiplicity of actions within the cells. Its participation in time-dependent feedback loops involving other ions (e.g. Na^+ and K^+) and second messengers (e.g. cyclic AMP and cyclic GMP), and its heterogeneous and variable compartmentalization within the muscle fibre (Rasmussen & Goodman, 1977), complicate precise interpretation of experimental data. However, the observed changes in the muscle's calcium content (e.g. Fig. 3) were in keeping with the generally accepted mode of action of such agents in increasing the concentration of cytosolic Ca^{2+}. In addition, in the present study some possible alternative explanations of our findings have been investigated and largely eliminated. Such possible alternative coupling agents as cyclic AMP and cyclic GMP, which are known to increase in response to influxes of Ca^{2+} (Rasmussen & Goodman, 1977), induced precisely the opposite changes in protein synthesis (Table 3) to that of Ca^{2+} (Fig. 1; Tables 1 and 2). Hence it is unlikely that these cyclic nucleotides mediated the observed responses. Not all previous studies are, however, in complete agreement with our findings (Beatty & Bocek, 1970). In addition, although Ca^{2+} may act as a second messenger in some hormonal responses, it seems highly improbable that this cation directly couples insulin's regulation of protein metabolism in muscle. Physiological concentrations of insulin induced purely anabolic changes (Table 3), compared with the strongly catabolic changes produced by Ca^{2+} (e.g. Table 2). Similarly, our findings are not consistent with a simple direct coupling of Ca^{2+} and protein turnover as an explanation of the increased rates of both synthesis and breakdown in dystrophic muscle (Simon *et al.*, 1962) and mechanically overloaded muscles (Goldspink, 1977; Laurent *et al.*, 1978).

Finally, an important implication arising from the present and other studies (Lewis & Goldspink, 1982) is that changes in the ionic composition of the internal milieu of cells can profoundly affect the measured rates of protein synthesis and protein breakdown. Such effects may be relevant to studies not only on isolated muscles incubated *in vitro*, but on perfused tissue and hemicorpus preparations also, where the normal distribution of ions between the tissue and the plasma (i.e. perfusate) may not be fully maintained (V. R. Preedy, R. Ross & P. J. Garlick, personal communication).

We thank Mrs. C. P. Verner and Mrs. G. Sanderson for their excellent technical assistance and the Fund for Research into Crippling Diseases for their financial support. S. E. M. L. is a Clinical Research Fellow of the Department of Health and Social Services, Northern Ireland.

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