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Action of Insulin and Cell Calcium: Effect of Ionophore A23187

S. Grinstein and D. Erlij*

Departamento de Fisiología, Centro de Investigación, I.P.N., México 14, D. F., México

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Summary. We have measured the effects of the carboxylic Ca^{++} ionophore A23187 on muscle tension, resting potential and 3-O-methylglucose efflux. The ionophore produces an increase in tension that is dependent on external Ca^{++} concentration since (a) the contracture was blocked by removing external Ca^{++} and (b) its size was increased by raising outside Ca^{++}. Neither resting potential nor resting and insulin-stimulated sugar efflux were modified by the ionophore. These data imply that the action of insulin is not mediated by increasing cytoplasmic [Ca^{++}]. Additional support for this conclusion was obtained by testing the effects of caffeine on sugar efflux. This agent, which releases Ca^{++} from the reticulum, did not increase resting sugar efflux and inhibited the insulin-stimulated efflux. Incubation in solutions containing butyrated derivatives of cyclic AMP or cyclic GMP plus theophylline did not modify the effects of insulin on sugar efflux. Evidence suggesting that our experimental conditions increased the cytoplasmic cyclic AMP activity was obtained.

One of the useful features of antibiotics that translocate ions across membranes is that they can serve to modify the premeability of biological membranes and the gradients of concentration and potential across them. This property can be used to verify theories that ascribe physiological changes to modifications of cell ion concentration or membrane potential (McLaughlin & Eisenberg, 1975).

In this study we have investigated the actions of the carboxylic calcium ionophore A23187 on the membrane potential and tension of rat skeletal muscle cells. Our findings suggest that, in line with its known selectivity for divalent cations, (Reed, 1972; Case, VanderKooi & Scarpa, 1974), the antibiotic enhances the entry of Ca from the outside solution into the muscle cytoplasm. We have used this property to test an hypothesis (Clausen, Elbrink & Dahl-Hansen, 1975; Elbrink & Bihler, 1975; Kissebah,

*Reprint requests: Dr. D. Erlij, Department of Physiology, SUNY Downstate Medical Center, 450 Clarkson Ave., Brooklyn, N.Y. 11203.

Hope-Gill, Vydelingum, Tulloch, Clarke & Fraser, 1975) that postulates that the action of insulin on cell membranes is mediated by increases in cytoplasmic calcium.

One of the reasons to propose a mediator for insulin action is the need to explain how the combination of a relatively low number of hormone molecules with membrane receptors will affect a variety of cell functions. These include sugar (Levine, Goldstein, Klein & Huddlestun, 1949), aminoacid (Kipnis & Noall, 1958; Narahara & Holloszy, 1974), and alkali cation transport (Gourley, 1965; Grinstein & Erlij, 1974), glycogen (Larner, Villar Palasi, Goldberg, Bishop, Huijing, Wenger, Sasko & Brown, 1968) and protein synthesis (Wool, Castles, Leader & Fox, 1972) and lipolysis (Ball & Jungas, 1964).

Calcium is not the sole candidate postulated as a mediator of insulin action. Thus, it has also been suggested that an increase in cyclic GMP concentration (Hollenberg & Cuatrecasas, 1975), a decrease in cyclic AMP concentration (Exton, Lewis, Ho, Robison & Park, 1971), or an increase in cell Mg^{++} (Lostroh & Krahl, 1973) mediate the effects of insulin.

In addition to carrying out experiments to test whether Ca^{++} can be considered as a mediator for insulin, we have also explored the possibility that changes in the levels of either cyclic AMP or GMP may mediate the action of the hormone.

Materials and Methods

Small rats weighing 60 g or less were anesthetized by intraperitoneal injection of Nembutal (35 mg/kg). Both soleus muscles were rapidly isolated, taking care not to interfere with the blood supply until the end of the dissection.

The muscles were mounted at resting length in a holder that was placed in a chamber that contained Ringer's solution bubbled with 95% O_2 – 5% CO_2; the temperature was controlled at 30 °C. To obtain the tension tracings, a Grass mechanoelectrical transducer connected to a Grass polygraph was used.

To record resting potentials, micropipettes filled with 3 M KCl (resistances 10 to 20 MΩ) and with tip potentials of 5 mV or less were used. The pipettes were connected to a high input impedance amplifier (Bioelectric Instruments Inc.) through Ag – AgCl electrodes. The output of the amplifier was recorded on an ink writing recorder.

The muscles were stimulated with square pulses (duration 0.5 msec) generated by a Grass stimulator. The intensity was adjusted to 1.5 times that giving maximal twitch response.

Isotope fluxes

The muscles were loaded for 1 hr in Ringer's solution at 30 °C containing 0.5 mM 3-O-methylglucose (Sigma) and 8 μ Ci/ml ^{3}H-3-O-methylglucose (New England Nuclear).

After washing the extracellular radioactivity by incubating the muscles for one additional hr in nonlabeled Ringer's at 0 °C, efflux was determined by transferring the muscles (at given time intervals) through a series of tubes containing Ringer's solution at 30 °C. Stirring was provided by a fine stream of 95% O_2 – 5% CO_2. The muscles were then minced and digested in NCS tissue solubilizer. Radioactivity was counted in a Packard Tri-Carb liquid scinitillation Spectrometer and quenching was determined by external standardization. From the backaddition of the radioactivity in the efflux samples to the radioactivity remaining in the muscle, we calculated the efflux rate constant, i.e., the fraction of the total radioactivity lost per unit time.

The uptake of ^{3}H-dibutyryl cyclic AMP was determined by a kinetic method (*see* Results). The muscles were incubated for 40 min at 30 °C in solutions containing 10^{-4} M N^6O^2 dibutyryl cyclic AMP (Sigma) and 2 μ Ci/ml ^{3}H-dibutyryl cyclic AMP (New England Nuclear). Washout was carried out at 3 °C immediately after loading, as described above.

Phosphorylase activity

Phosphorylase activity was determined essentially as described by Danforth, Helmereich & Cori (1962). Paired muscles were used to provide control and experimental samples. For each determination two muscles were used. After incubation in the appropriate solution, the muscles were minced and homogenized in 30 volumes of a solution containing (in mmoles/liter): NaF 20; sodium glycerophosphate 20; L-cystein 20; EDTA 1.0, pH 6.7. After centrifugation (5000 × *g* for 5 min) the supernatant was diluted twofold with the homogenizing solution.

An aliquot (0.2 ml) of the dilute supernatant was incubated for 30 min at 37 °C in the presence of 0.1 ml of an assay solution containing 2% glycogen, 32 mM glucose-1-phosphate with or without 1 mM 5′ AMP. The reaction was stopped by the addition of 1 ml of ice-cold 0.1 M acetate buffer pH 4.0. Inorganic phosphate was determined as described by Bonting, Simon & Hawkins (1962). The results were expressed as the ratio of the phosphorylase activity found in the assay without 5′ AMP over that found in the presence of the nucleotide. This ratio is proportional to the percentage of phosphorylase *a*.

Ringer's solution had the following composition (in mmoles/liter): NaCl, 118; KCl, 5.0; $CaCl_2$, 1.2; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; pyruvate, 1. This solution was equilibrated with a gas mixture of 95% O_2, 5% CO_2. In the majority of the experiments curare (10^{-4} M) was added to Ringer's solution.

A23187 was dissolved in absolute alcohol and added to the Ringer's solution to give a final ethanol concentration of 0.5% (v/v).

Results

Effects of A23187 on Muscle Tension and Resting Potential

Although it is not easy to determine the free calcium concentration in the muscle cytoplasm, development of tension by the muscle constitutes a sensitive indicator of increases in intracytoplasmic Ca; when intracellular Ca concentration increases beyond a level of about 5×10^{-7} M, the contractile system of the muscle is activated and tension is developed (Hellam & Podolsky, 1968).

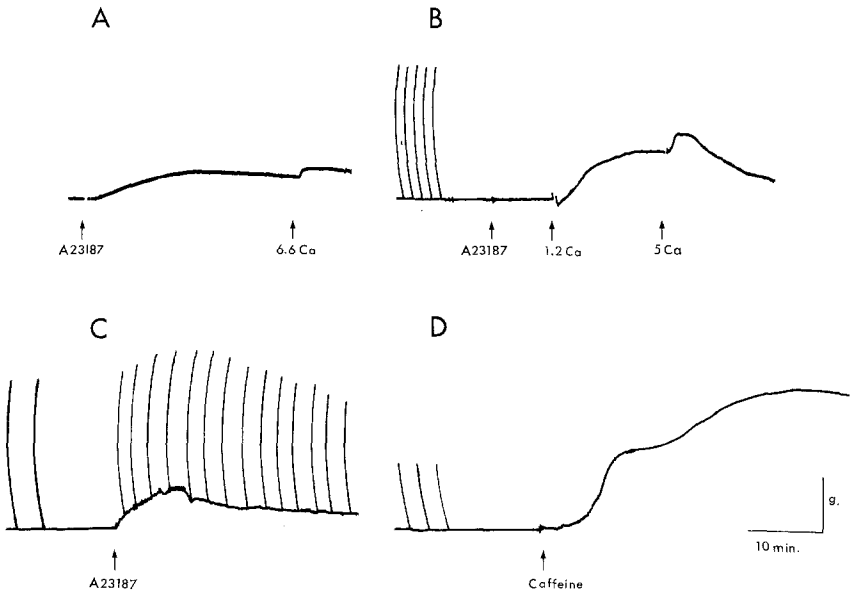

Fig. 1. The effects of A23187, extracellular calcium, and caffeine on muscle tension. (*A*) The effects of 10 μg/ml of A23187 and of increasing external Ca^{++}. (*B*) A muscle equilibrated in Ca-free Ringer's was stimulated 5 times, then A23187 (10 μg/ml) was added, and finally increasing concentrations of Ca were added to the solution. (*C*) The effects of A23187 (10 μg/ml) on twitch tension. (*D*) Caffeine (8 mM) was added at the moment indicated by the arrow. The Ca^{++} concentrations are given in mmoles/liter. The horizontal bar is the time calibration for all records. The vertical bar is the tension calibration: *A*, $2 \times g$; *B*, $0.5 \times g$; *C*, $1 \times g$; *D*, $2.5 \times g$

Fig. 1*A* shows that addition of 10 μg/ml of the antibiotic A23187 to a Ringer's solution containing 1.2 mM Ca caused an increase in resting tension of the rat soleus muscle. The tension increased slowly, reaching a maximum between 5 and 10 min after the addition of the antibiotic. After the antibiotic-induced contracture reached a maximum level, the tension remained constant for variable periods of time (4 to 10 min), and then spontaneously decayed. When expressed as a fraction of the tension developed by a single maximal twitch, the increase in tension caused by A23187 in 8 muscles had an average value of 26.75% (SE = 1.75%). When a concentration of A23187 of 20 μg/ml was used in other muscles, the increase in tension was 29% ($n = 3$) of the maximum single twitch.

Fig. 1*A* also shows that the concentration of extracellular Ca can influence the tension induced by the addition of A23187. When Ca in the bathing solution was increased from 1.2 to 6.6 mM a small but clearcut increase in tension was always observed.

To further explore the dependency of the action of A23187 on extracellular Ca concentration, we carried out experiments in which the muscles were immersed in Ca-free solutions for 30 min before starting the experiment. The initial part of the experiment was also carried out in a Ca-free solution. One such experiment is shown in Fig. 1*B*. In the first part of the figure, the muscle was stimulated to give 5 maximal single twitches. In agreement with previous observations, immersion in nominally Ca-free solutions did not suppress mechanical activity of the muscle since external Ca^{++} seems to play no essential part in action potential propagation or excitation-contraction coupling in skeletal muscle (Armstrong, Bezanilla & Horowicz, 1972). In contrast, A23187 had no effects on tension when added to the Ca-free solution. However, when the Ca-free solution was replaced by Ringer's with 1.2 mM of Ca, the antibiotic-induced contracture was produced. Fig. 1*B* shows again that increasing Ca concentration above 1.2 mM causes further increases in tension. In a group of control experiments we found that the addition of 1.2 mM Ca to muscles bathed in Ca^{+}-free Ringer's did not produce a contracture if the muscles were not pretreated with the antibiotic.

Fig. 1*C* illustrates one of four experiments carried out to discover whether A23187 has any effect on the amplitude of the muscle twitch. This question arose from observations of barnacle muscle fibers (Hainaut & Desmedt, 1974), where A23187 does not modify resting tension although it markedly increases twitch tension. In these experiments the antibiotic was added after determining the amplitude of the maximum twitch. Then the muscle was stimulated in the presence of the antibiotic. In this situation, twitch tension was measured using the contracture tension as the baseline. During the rising phase and the sustained peak of the contracture, twitch tension was the same as during the control period. When the contracture began to decline, the twitch decreased in parallel to levels as low as 55% of the control value.

Recent experiments in frog sartorius muscle (Devore & Nastuk, 1975) have shown that the effects of X537A, an antibiotic that has been widely used to modify Ca permeability, are probably mediated by an increase in Na permeability rather than by a direct enhancement of Ca permeability. Devore and Nastuk (1975) found that X537A produced a depolarization that was dependent on the presence of Na in the bathing medium. For this reason, it was important to determine whether the contracture induced by A23187 in our experiments was the result of depolarization. Our observation that muscles remain excitable during the action of A23187 already suggests that the resting potential is not drastically

altered. Indeed, when we measured directly the resting potential we found an average value of 87.27 mV $\pm$ 0.91 ($n=52$) in control muscles, while during treatment with 10 μg/ml of A23187 the average value was 85.12 $\pm$ 0.72 mV ($n=83$). These measurements show that the A23187-induced contracture is not triggered by membrane depolarization.

Fig. 1*D* illustrates one of the experiments carried out to compare the effects of A23187 with those of caffeine. The latter substance produces a contracture which is the result of Ca release from the sarcoplasmic reticulum (Weber & Herz, 1968).

After a latency that varied between 5 and 12 min, the addition of 8 mM caffeine caused a slowly developing contracture that reached a level of 1.8 times ($n=5$) the tension produced by the maximum single twitch.

Effects on Sugar Efflux

To test whether the insulin-induced changes of membrane permeability are mediated by an increase in cytoplasmic Ca we tested the effects of A23187 on sugar efflux. The enhancement of sugar permeability is one of the most characteristic effects of insulin on the cell. Therefore, we selected the sugar efflux from rat soleus muscle as a parameter to assay the action of agents that modify cell Ca. Although the effects of insulin have been more widely studied on sugar influx, it is now well established that the efflux of nonmetabolizable sugars from rat muscle is markedly stimulated by insulin (Kohn & Clausen, 1971). In addition to sharing many features with the enhancement of sugar influx, the study of the efflux has an advantage in that the time course of succesive experimental modifications can be followed in the same muscle.

A23187. Fig. 2 illustrates one of five experiments in which the effects of A23187 and insulin on the efflux of 3-O-methylglucose from rat soleus muscles were studied succesively. During the control period the rate constant for sugar efflux declined, approaching in the long run a steady level that in 18 muscles averaged 0.0080 $\pm$ 0.0006 min^{-1}. After the control period the efflux of the test muscle was measured in a solution containing 10 μg/ml of A23187, while the control muscle was transferred through a series of tubes to which only an amount of ethanol equal to that used as a vehicle for the antibiotic had been added. Neither the antibiotic nor ethanol had any significant effects on sugar efflux.

In the final period both muscles were treated with insulin (250 mU/ml). The insulin-containing solution used for measuring efflux from

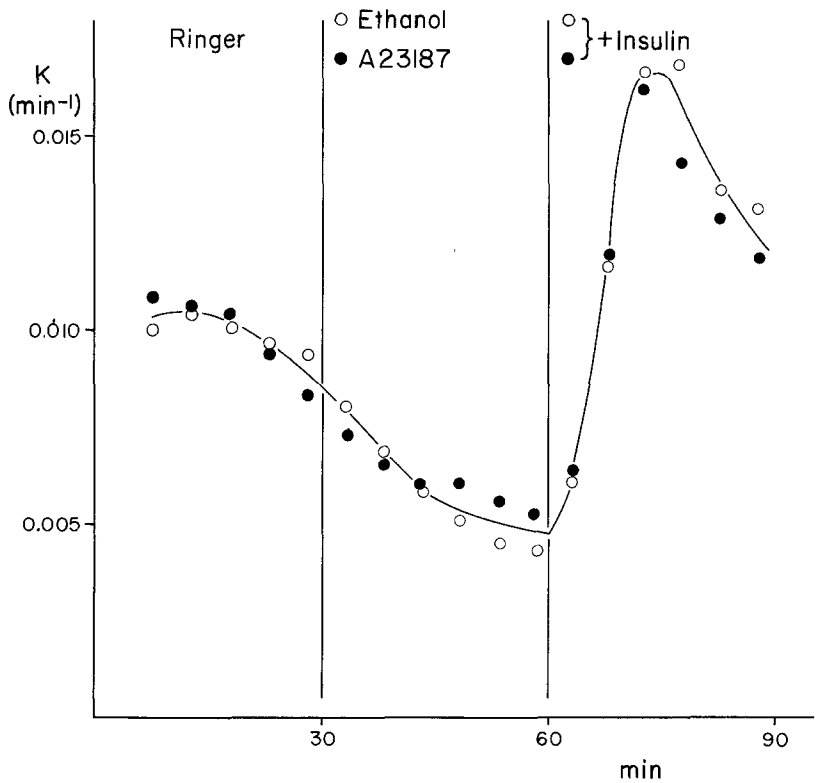

Fig. 2. Effects of A23187 on the efflux of 3-O-methylglucose. After an initial period of efflux, the experimental muscle (filled circles) was treated with 10 μg/ml of A23187 while the control muscle (empty circles) was treated with ethanol (0.5% v/v). Finally insulin (250 mU/ml) was added to both muscles. *Ordinate:* fraction of total sugar in muscle lost per min

the test muscle also contained 10 μg/ml of A23187. As observed by other workers (Kohn & Clausen, 1971), insulin caused a rapid and transient increase in sugar efflux. The efflux was increased to 3.44 ± 0.45 times the control level ($n = 10$). The increases were of similar magnitude in both the control and the A23187 treated muscles.

Caffeine. The action of caffeine on sugar efflux of rat soleus muscle and its effects on the stimulation of sugar efflux caused by insulin are illustrated in Fig. 3.

After a control period the efflux of 3-O-methylglucose of the test muscle was followed in solutions containing 8 mM caffeine. In some experiments caffeine produced a small (about 20%) decrease in the rate constant of the efflux, while in other instances it had no detectable effects.

As shown in Fig. 3, caffeine markedly reduced and delayed the stimu-

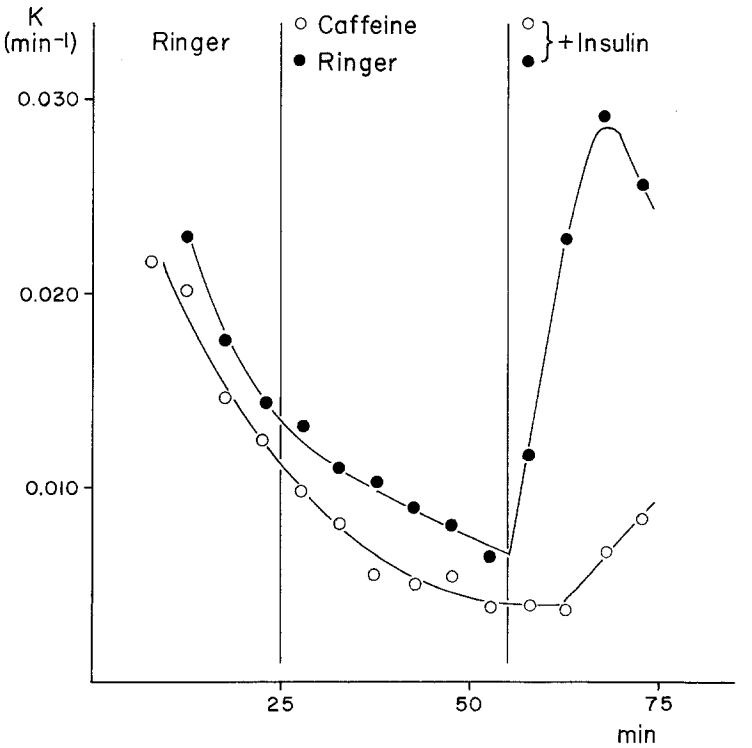

Fig. 3. Effects of caffeine on 3-O-methylglucose efflux. After the control period the experimental muscle was treated with 8 mM caffeine and finally both muscles received insulin (250 mU/ml). *Ordinate:* Fraction of total sugar in muscle lost per min

lation of sugar efflux caused by insulin. Instead of the prompt rise caused by insulin in control experiments, in the presence of caffeine only a slow and delayed stimulation was observed that, during the first 20 min, never reached more than 30% of the control effect. Although these results are in agreement with the recent report of Clausen *et al.* (1975) carried out under similar experimental conditions, they differ from the observations of Holloszy and Narahara (1967), who found that caffeine (3 mM) increased sugar uptake in frog sartorius muscle.

Cyclic Nucleotides. It has been postulated that insulin effects are mediated by a reduction in cyclic AMP levels within the cell (Exton *et al.*, 1971; Hollenberg & Cuatrecasas, 1975). Since caffeine inhibits the phosphodiesterase that hydrolizes cyclic AMP and raises intracellular levels of the nucleotide, its inhibitory effect on the insulin induced stimulation of sugar efflux may be related to increases in the level of cyclic AMP. To find whether increased intracellular levels of cyclic AMP could indeed interfere with the action of insulin, we measured the action of

the hormone on sugar efflux under conditions in which the cellular levels of the nucleotide were artificially increased. In our experiments we tested the effects of 10^{-4} M of N^6 monobutyryl 3′, 5′-cyclic AMP and of N^6-O^2 dibutyryl 3′, 5′-cyclic AMP either in the presence or absence of theophylline (0.5 mM).

Fig. 4*A* shows one experiment in which monobutyryl cyclic AMP was given. No effects were observed either on the resting or the insulin-stimulated sugar efflux. Similar lack of effects were found when either nucleotide was given alone or associated with 0.5 mM theophylline.

Another cyclic nucleotide that has been implicated in the action of insulin is cyclic GMP since its concentration has been found increased during the action of insulin (Illiano, Tell, Siegel & Cuatrecasas, 1973). Fig. 4*B* illustrates one of the experiments in which 10^{-5} of the N_2 monobutyryl derivative of cyclic GMP was tested. As in the case for cyclic AMP, cyclic GMP was without effects on the sugar efflux and on its stimulation by insulin.

To establish that the cytoplasmic activity of cyclic nucleotides was increased under our experimental conditions, we carried out two types of experiments. First we determined whether these compounds penetrated the cell membrane. Fig. 5 illustrates one of seven experiments carried out to determine the penetration of N^6, O^2 dibutyryl 3′ 5′ cyclic AMP into the rat soleus muscle. After equilibrating the muscle for 40 min. in a solution containing 10^{-4} M of the tritiated nucleotide, the efflux of radioactivity into a nucleotide-free solution was followed. Following an initial period of rapid efflux, the amount of label remaining in the muscle decreased less steeply. As illustrated in Fig. 5, graphical analysis of the results showed that the efflux process is made up by the sum of two exponential components. This analysis, taken together with previous measurements of washout of isotopes from skeletal muscle (Harris, 1963), suggests that the fast component corresponds to washout from the extracellular space and that the slow component corresponds to the intracellular space. The analysis shows that more than 95% of the radioactivity in the extracellular space was leached after 50 min of washing at 3 °C. The size of this extracellular space had an average value of 0.20 ml/g ($n=7$) which is in general agreement with previous determinations in skeletal muscle (Harris, 1963).

The intercept of the slow component—which provides an estimate of the amount of radioactivity in the intracellular space—was equal to $1.33\times10^{-5}\pm0.01$ moles/liter of cell water of the original labelled dibutyryl cyclic AMP. It is likely, however, that an important fraction

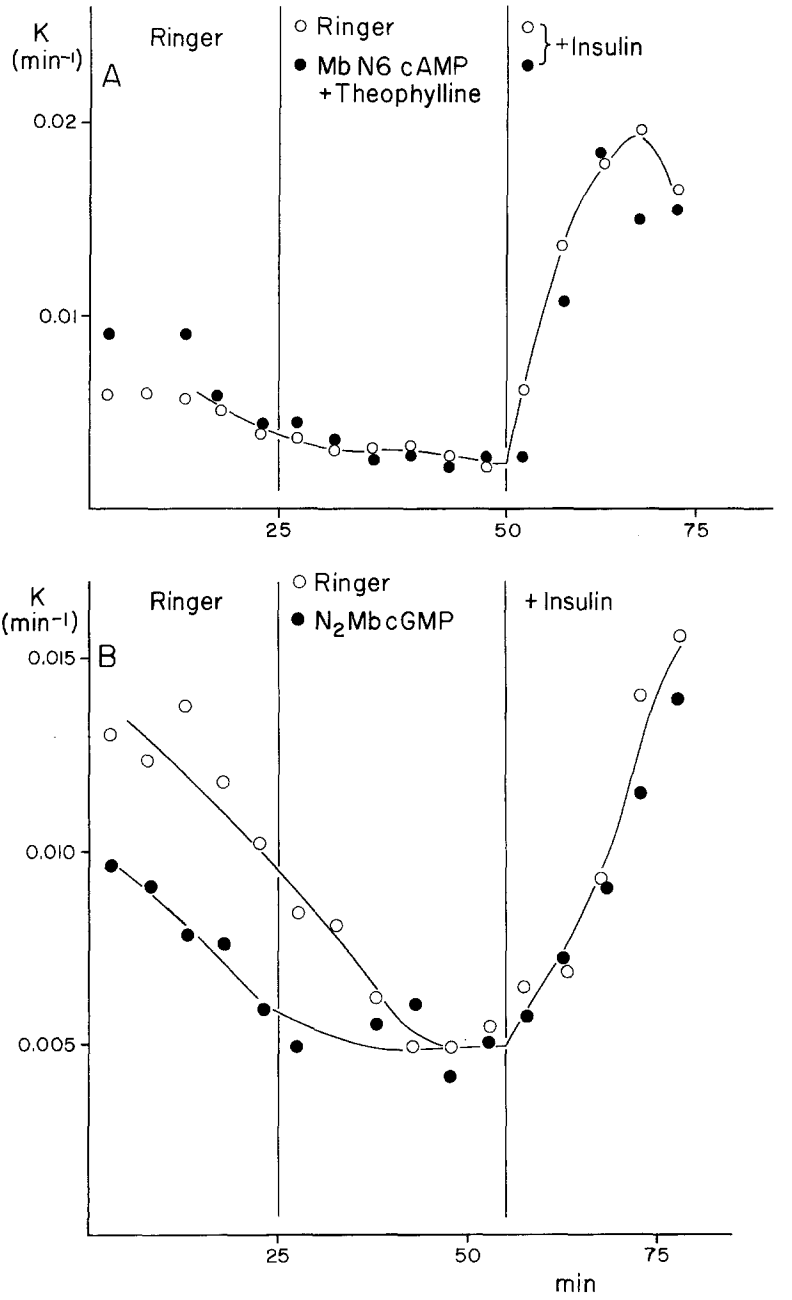

Fig. 4. The effects of cyclic nucleotides on 3-O-methylglucose efflux. (*A*) The effects of N6 monobutyryl cyclic AMP (10^{-4} M) + theophylline (0.5 mM) on the resting and insulinstimulated sugar efflux. (*B*) Effect of N2 monobutyryl cyclic GMP (10^{-5} M) on the resting and insulin-stimulated sugar efflux. *Ordinate:* fraction of total sugar in muscle lost per min

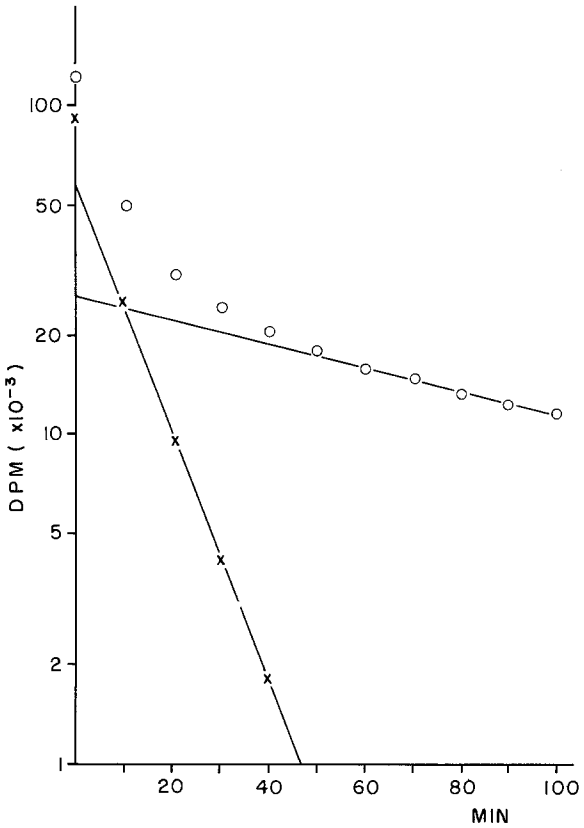

Fig. 5. Washout of $^3H-N^6$, O_2-dibutyryl cyclic AMP from the soleus muscle. The ordinate is the total amount of radioactivity remaining in the muscle at any time. The crosses were calculated by subtracting the slow exponential from the total curve. The sum of the extrapolated intercepts do not add to the total radioactivity. This is probably due to the radioactive material adhering to the surface of the muscle and to the holder, which is washed in the first efflux period

of the radioactivity may be present as N^6 monobutyryl cyclic AMP since Swislocki (1970) found that under the same experimental conditions about 20% of dibutyryl cyclic AMP was degraded to the monobutyrated derivative, which very likely has a greater activity than dibutyryl cyclic AMP itself (Kaukel, Mudhenk & Hinz, 1972; Neelon & Birch, 1973). To further test whether the selected experimental conditions increased cyclic AMP activity inside the muscle, we compared phosphorylase activity in paired control and treated muscles. The experimental muscles were incubated for 40 min in medium containing 10^{-4} M of either dibutyryl or monobutyryl cyclic AMP and 0.5 mM theophylline while the control remained in Krebs Ringer's solution. To insure that the agents in the incubation medium would not affect the assays, the muscles were leached

for 40 min in ice-cold Ringer's before homogenization. As shown in Fig. 5, after this period more than 95% of the extracellular space had been washed while only a small fraction of the intracellular nucleotide was lost. Both treatments increased phosphorylase activity as evidenced by the increase in the $-AMP/+AMP$ activity ratio (Danforth *et al.*, 1962). The ratios in the presence of mono and dibutyryl cyclic AMP were 0.318 ± 0.05 and 0.330 ± 0.03, respectively, while the ratio in control muscles was 0.135 ± 0.03 ($n=7$).

Discussion

The first point to emerge from our experiments is that A23187 produces an increase in cytoplasmic Ca^{++} which is probably due to the influx of extracellular Ca^{++} mediated by the antibiotic. This conclusion is based on the observation that the increased tension caused by A23187 is dependent on the [Ca] in the extracellular solution; the contracture was blocked by removing external Ca, and its size was increased by raising extracellular [Ca]. A23187-induced contractures that depend on external calcium have also been observed in the smooth muscle of the aorta and the gut. (Pressman, 1972; Triggle, Grant & Triggle, 1975).

Further support for the notion that the A23187-induced contracture is mainly the result of Ca influx is provided by the finding that the antibiotic did not modify the resting potential across the muscle membrane. This observation is of interest in view of the doubts cast on the interpretation of findings made with another carboxylic antibiotic, X537A. Although it had been concluded that a number of physiological effects caused by X537A are a result of enhanced Ca movements mediated by the antibiotic, recent observations (Devore & Nastuk, 1975) show that this agent causes a depolarization of frog skeletal muscle mediated by an increase in Na permeability. Some of the effects of the antibiotic can then be attributed to increased Na permeability and depolarization, rather than to a direct enhacement of Ca permeability. Our results with A23187, taken together with its greater selectivity for divalent cations (Reed & Lardy, 1972), show that it provides a more reliable tool than X537A for enhancing divalent cation movements across biological membranes.

The second point to be considered bears on the identity of a possible mediator for the action of insulin. To conclude that the action of a hormone is mediated by a change in the intracellular concentration of

a given substance, a number of criteria ought to be fullfilled. These include at least the following:

1. Addition of the hormone must produce the appropriate changes in the intracellular concentration of the purported mediator.

2. In the absence of the hormone, appropriate changes in the intracellular concentration of the mediator must imitate the effects of the hormone.

3. Hormonal action must be blocked when changes in the concentration of the postulated mediator are impeded.

The finding that A23187 did not modify either the resting or hormone stimulated sugar efflux shows that increases in cytoplasmic Ca that reach the level to activate the contractile machinery of the muscle do not mimic the action of insulin nor interfere with its action. A further point that suggests that the action of insulin is not mediated by an increase in cytoplasmic Ca, is that the addition of the hormone does not modify the tension of control muscles (Clausen *et al.*, 1975).

Additional evidence against the role of calcium as a mediator for insulin action comes from the experiments in which we measured the effects of caffeine on sugar efflux. This agent released a large amount of Ca from the sarcoplasmic reticulum without causing any increase in sugar efflux.

Chambaut, Eboué-Bonis, Hanoune & Clauser, (1969) found that the insulin-induced increase in sugar uptake was blocked when theophylline and dibutyryl cyclic AMP were given together, while theophylline alone had no effect on either the resting or the insulin-stimulated sugar uptake.

In contrast, we could not detect any effects of cyclic nucleotides given together with theophylline on the action of insulin. The finding that conditions that increase cyclic AMP activity within the cell do not block insulin action suggests that it is unlikely that a reduction in the overall levels of intracellular cyclic AMP mediated the increase in sugar movements. Another finding in disagreement with the cyclic AMP reduction hypothesis is that insulin does not decrease 3′ 5′ cyclic AMP levels in skeletal or cardiac muscle (Goldberg, Villar-Palasi, Sasko & Larner, 1967; Keely, Corbin & Park, 1975) even though these organs are among the most conspicuous targets for insulin action.

We did not obtain any evidence indicating that cyclic GMP derivatives penetrate into the muscle cell. However, findings in other systems (McAfee & Greengard, 1972; Stone, Taylor & Bloom, 1975) indicate that exogenous GMP penetrates into cells. These results, taken together with the evidence that indicates that butyrated nucleotides penetrate cell mem-

branes, suggest that in our experiments there probably was an increase in cyclic GMP derivatives within the cell as it was shown for the AMP derivatives. Provided these considerations are correct, the lack of effects of cyclic GMP on sugar efflux are not in agreement with the hypothesis (Hollenberg & Cuatrecasas, 1975) that suggests that increases in the overall cytoplasmic levels of this nucleotide mediate the action of insulin.

Although the experiments in which the nucleotide levels were artificially modified provide arguments against the theories that suggest that changes in overall levels of these substances act as universal mediators of insulin action, they do not rule out the possibility that only some actions of insulin may be mediated by overall changes in nucleotide concentration, nor that the hormone modifies nucleotide levels in a particular cell compartment. The finding that there is a compartmentalization of 3′ 5′ cyclic AMP in several cell types (Ong, Whitley, Stowe & Steiner, 1975) suggests that the latter proposal is not improbable.

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