Section of Endocrinology

President G F Joplin FRCP

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Guest Lecture

Is Insulin's Second Messenger Calcium?[1]

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It is now over fifty years since Banting and Best's discovery of insulin transformed the treatment of diabetes mellitus. But the long-term control of diabetes still remains a problem, stimulating study of the control of insulin's secretion as well as of factors affecting the sensitivity to insulin. Over the last few years, therefore, our unit has been studying tissue sensitivity to insulin, from which has emerged evidence offering a clearer picture of how insulin may act on cells. Before outlining these findings, I wish to acknowledge my indebtedness for these data to several colleagues who have produced it in our laboratories, especially to Dr A Kissebah, Dr B Tulloch, Dr N Vydelingum, Dr H Hope-Gill and Dr Peter Clarke.

Insulin's known biological actions: Insulin was from the start found to lower blood sugar and to reverse all the features of diabetes mellitus. But what is its normal function, and how does it achieve its effects on cells? Food intake is the main stimulus to its secretion and it effects storage of foodstuffs against their later usage in starvation. For the whole body, it is evidently an anabolic hormone which promotes synthesis and storage of glycogen, triglyceride and protein. Concurrently it is antilipolytic, antiglycogenolytic and antigluconeogenic.

At the cells, it binds to a specific surface receptor whence it induces its main effects without entering the cell (Cuatrecases 1972). One of the first of these effects noted was its increase of the cell's uptake of glucose. For example, Soskin & Levine (1937) showed that in hepatectomized dogs normal utilization of glucose is achieved from a lower blood sugar level in the presence of insulin than without insulin. Further, studies by Park (Morgan *et al.* 1960) with the isolated heart have shown how quickly insulin increases the cellular uptake of glucose – within minutes; and that it does this less effectively with diabetic tissue.

More recently, with the development of techniques for isolation of fat and liver cells a demonstrable effect of insulin on glucose and amino acid transport has been substantiated.

However, insulin also induces within the cells several enzymatic changes demonstrable even with *in vitro* studies where the incubate contains no substrate whose uptake insulin could increase. Insulin first affects 'kinase-type' enzymes, i.e. those which either activate or inactivate other enzymes, as illustrated by its promoting in the liver activation of the enzymes of glycogenesis and inhibition of those of glycogenolysis. Similarly in adipose tissue insulin stimulates key regulatory enzymes which activate lipogenesis and inhibit lipolysis (Weber *et al.* 1966). So we seem to have many cellular sites for effects induced by this hormone, which does not enter the cell – surely it must have a second messenger.

Theories: When Butcher *et al.* (1966) proposed cyclic adenosine monophosphate (cAMP) to be the second messenger for the actions of adrenaline and glucagon on the liver, this was soon found to be the mechanism of action of many hormones – but not of all, and insulin particularly has re-

[1]Adapted from the Claude Bernard Lecture given to the European Diabetic Association, 1974

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mained an enigma. Initial reports suggested that it may achieve its effects by lowering cAMP by altering phosphodiesterase or adenyl cyclase. However, recent studies (Fain & Rosenberg 1972, Miller & Larner 1973, Walaas *et al.* 1974), including our own as we shall see, have revealed at most a poor correlation between the extent of the cAMP changes which insulin causes and its familiar cellular effects.

What alternative hypotheses suggest themselves? (1) Insulin could alter the enzymic responses to cAMP; it could be antilipolytic by impairing cAMP's activation of the phosphorylating protein-kinase. Thus, it might intervene and oppose the cascade of enzymic effects which cAMP causes – e.g. those leading to increased lipolysis, or those leading to glycogenolysis. It could also promote dephosphorylation, by activating a phosphatase, and so counteract as well as antagonize the effects of cAMP's activation of the protein-kinase, which phosphorylates dependent enzymes. (2) It might do this by releasing at the cell plasma membrane another second messenger, opposite in its effects to cAMP.

The following evidence suggests that both of these hypotheses apply. It seems reasonable that for adequate hormonal control a cell needs both an activator mechanism and an inhibitor mechanism. The fat cell needs one mechanism which can induce lipolysis, and another which can induce instead lipogenesis, while each counters the other's effects.

Methods

Our experiments have been largely *in vitro* studies which use the isolated fat cell (Rodbell 1964) as a model for studying insulin's action. Insulin's antilipolytic effect has been chosen for measuring the action of insulin, since this effect is the cell's most sensitive response to insulin (Fain *et al.* 1966) and it is also conveniently measurable.

Fig 1 shows an illustrative experiment, which defines the pattern used in most of our studies (Kissebah, Tulloch *et al.* 1974). Incubates of isolated rat epididymal fat cells have been sampled at intervals to measure the responses seen after adding to the incubate the chosen hormones or drugs. The control tubes usually contain cells in standard Krebs buffer with adrenaline added to stimulate their lipolysis; and the experimental tubes (dotted lines) contain cells in this buffer with adrenaline, as well as additions of either insulin or of a drug with which insulin

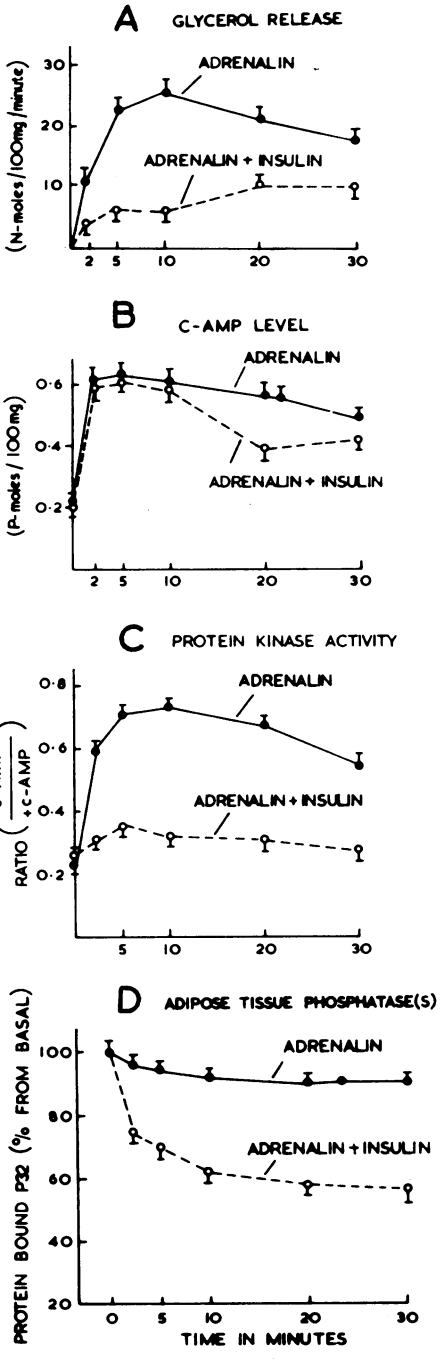

Fig 1 *Time curves comparing* A, *glycerol release;* B, *cAMP levels;* C, *protein-kinase activity;* D, *phosphatase-like activity of rat adipocytes incubated with 100 μg/l adrenaline ± 100 mu/l insulin. (Mean ± s.e. mean of three experiments.) — adrenaline, – – – adrenaline + insulin. (Reproduced from Kissebah, Tulloch* et al. *1974, by kind permission)*

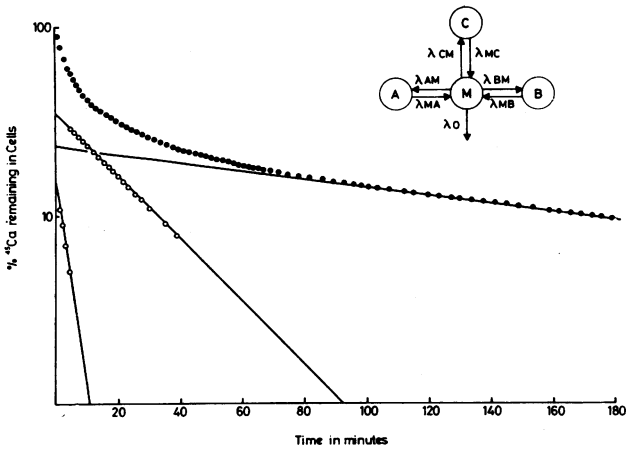

Fig 2 *'Washout' curve from perifused fat cells prelabelled with ^{45}Ca. Note inital rapid washout followed by steady normal efflux. (Perifused with Krebs albumin buffer including $CaCl_2$ 1.25 mM over three hours; sum of counts in each wash plus final residue in cells taken as 100%.) (Reproduced from Kissebah* et al. *1975, by kind permission)*

is being compared. Note that the insulin has prevented the lipolytic response to the adrenaline – yet we see in both sets of tubes equivalent cAMP early responses to the adrenaline – i.e. the insulin has hardly altered this adrenaline effect. Thus, while the insulin has clearly been anti-lipolytic, it has acted without altering the cell's cAMP response to adrenaline. Presumably it has done this by altering in some way the cascade of enzymic responses which are normally activated by the rise of cAMP levels.

The Hypothesis

Fig 8 shows a simplified scheme of how our experiments suggest insulin acts on the cell; for which we shall shortly see the supporting evidence. In Fig 8 we see an outline of the pathway controlling the cell's lipolysis. Thus, when adrenaline binds to its receptor, this releases cAMP which eventually activates the triglyceride lipase on the lipolytic pathway. Alternatively, when insulin binds to its receptor, then ionic calcium, also from the plasma membrane and/or endoplasmic reticulum, is released into the cytosol, where it counteracts the cAMP effects and so inactivates the triglyceride lipase, by dephosphorylation. Thus, ionic-calcium is postulated as the anabolic second messenger, both countering cAMP's effects and promoting the opposite effects such as lipogenesis. We may compare calcium's activating effects in other cells, such as its initiating muscular contraction, and its important involvement in the actions of neurotransmitters, and more relevantly, in the control of the secretion of insulin itself.

Is there calcium available in fat cells? We have found that isolated rat fat cells contain 262±11 nmol Ca per mg cell protein. As in other cells (Borle 1971), over 90% of this calcium is unexchangeable and probably forms part of the glycoprotein matrix of fat cell membranes. Thus we find that the uptake of ^{45}Ca by fat cells reaches isotopic equilibrium within 1–2 hours incubation. At that time the relative specific activity of calcium in cells to medium (which denotes the exchangeable calcium fraction) is about 9–10%. Moreover, the distribution of this exchangeable fraction is rather complex and involves three kinetically independent compartments. Fig 2 shows a representative experiment of the ^{45}Ca washout from prelabelled, perifused fat cells. When these curves are analysed using a multicompartmental model (shown top right), about 50% of the total exchangeable calcium seems to be bound to the plasma membrane since its release is not affected by metabolic inhibitors. The release of calcium from the second compartment, occurring at a rate which is similar to transmembrane fluxes in other cells, probably represents a cytoplasmic pool. The third compartment slowly exchanges with the second and probably represents a storage pool. This storage pool could be in the endoplasmic reticulum and/or in the plasma membrane. Fig 3 shows the binding affinity of fat cell ghosts to calcium. The Scatchard plot shows two distinct binding rates of high and low affinity which can accommodate up to 60 nmol Ca/mg protein.

Hales and his colleagues (Herman *et al.* 1973, Hales *et al.* 1974) have produced some impressive

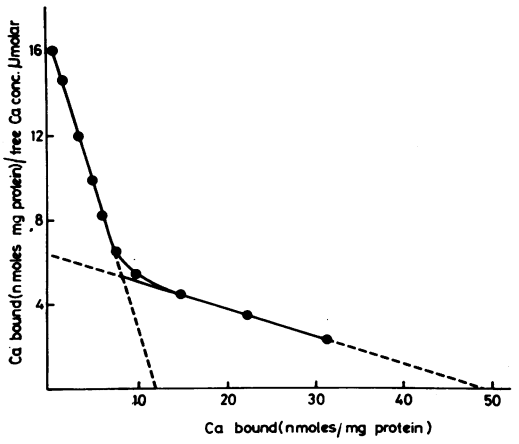

Fig 3 *Scatchard plot for calcium binding to fat cell ghosts. Fat cell ghosts were incubated with increasing concentrations of $CaCl_2$ from 10^{-7}M to 10^{-3}M with $^{45}CaCl_2$ 0.5 µCi/ml Bound calcium determined by Millipore filtration. Results are means of six determinations (Reproduced from Kissebah* et al. *1975, by kind permission)*

electron-microscopic evidence that within the fat cell calcium is concentrated in or near the plasma membrane. The sections for these have been prepared after precipitating the calcium as antimonate; the calcium shows as dark or electron-dense dots or lumps. In these fat cells a series of such calcium lumps are visible along the membrane edges but not diffusely in the cell. Hales has also used electron probe microanalysis to prove that these dark dots do indeed contain calcium. Thus, calcium stores are available in cells adjacent to the plasma membrane, waiting ready to be released into the cytosol and so to alter the set of the cell's activity.

From our desaturation experiments, the intracellular calcium would be approximately 0.3 mM/kg cell water. If a 1000-fold gradient exists, as has been suggested, between the endoplasmic reticulum, mitochondria and the cytosol it follows that the cytosol free calcium concentration would be approximately 10^{-6}M in fat cells. The plasma membrane of fat cells contains a calcium-sensitive adenosine triphosphatase which could maintain this gradient.

Our Evidence

(1) *Insulin has at most a minimal and late effect on the cell levels of cAMP, while nevertheless preventing the latter's lipolytic action:* Fig 1 shows measurements from an experiment of our standard type (Kissebah, Tulloch *et al.* 1974). In this, fat cells or shreds have been exposed to adrenaline with or without added insulin in the absence of theophylline. In Fig 1A we see that in these experiments the insulin does indeed have this full antilipolytic effect – it prevents the glycerol release induced by the adrenaline without added insulin. In Fig 1B we see that there is scarcely any difference in the cAMP levels between the two sets of tubes – perhaps a slight lowering after the insulin by the 20th minute, but the glycerol response was strikingly reduced at the 2nd and 5th minutes. This is hardly a precursor product relationship. Fig 1C gives us a hint of how the insulin achieves its antilipolytic effect; for this chart shows the state of activation of the protein-kinase which cAMP activates. The state of this enzyme's activation has been indexed by measuring the homogenate's phosphorylating activity – both that of untreated homogenate and also of homogenate to which enough cAMP has been added to achieve full activation. We see that in the 'adrenaline-only' tubes the protein-kinase has been rapidly activated to near maximal levels. However, in the 'adrenaline+insulin' tubes, this protein-kinase activation has remained low throughout the experiment. Thus, insulin seems to have interferred with cAMP's usual activation of this kinase – which enzyme would normally phosphorylate and so activate the lipase and increase lipolysis. Fig 1D shows measurements of phosphoprotein phosphatase activity in fat cell homogenates, which is found to correlate with the inactivation of the lipase. Here the adrenaline-stimulated control cells did not show dephosphorylating activity, but with homogenates from cells exposed to insulin as well as adrenaline, dephosphorylation proceeded rapidly.

(2) *Could these effects be due to calcium changes?* Purification and diethylaminoethanol column chromatography of fat cell homogenates, following the procedures of Huttunen & Steinberg (1971) and of Miyamoto *et al.* (1969), gave us a reasonably pure preparation of the protein-kinase and of the triglyceride lipase – as measured for the former by its cAMP binding and by its capacity to phosphorylate a histone. Using these enzyme preparations we have therefore examined the effects of calcium on the activation of a mixture of them (Kissebah, Vydelingum *et al.* 1974).

On adding cAMP with other required co-factors including 10^{-9}M Ca, these enzymes are well activated – as indexed both by the phosphorylation of the protein and by the release from triglyceride of ^{14}C-fatty acids. But with increasing concentrations of calcium up to 10^{-3}M, this activation is increasingly inhibited. And adding 10^{-3}M Ca to an already activated preparation is similarly inhibitory. On adding a protein kinase

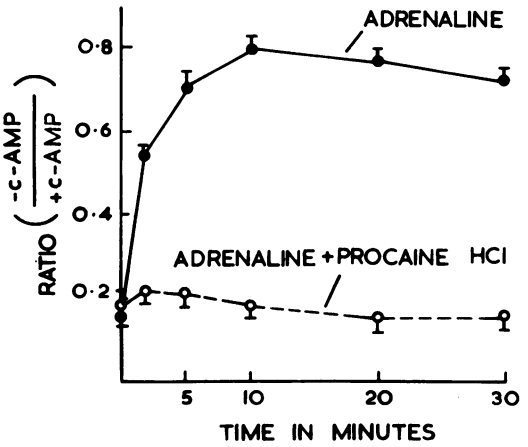

Fig 4 *Effect of procaine HCl on adrenaline-stimulated protein-kinase activity. Adipose tissue pieces were incubated with adrenaline 100 μg/l and procaine 1 mmol/l. Homogenates were prepared in the presence and absence of cAMP, and ^{32}P-protein-bound measured*

inhibitor to the activating mixture, rapid inactivation occurs, possibly due to the activity of a phosphoprotein phosphatase. After adding 10^{-3}M Ca, this phosphatase-like activity is increased. Thus 10^{-3}M Ca both prevents activation of the lipase and also deactivates it when already activated. We may note that the postulated 'normal' concentration of cytosol calcium is probably in the middle of this range (between 10^{-7}M and 10^{-5}M: Weber *et al.* 1964) – so these findings confirm the effects from changes of calcium concentration in the range postulated by our hypothesis.

(3) *Several other enzymic effects induced by insulin are also calcium activated:* Coore *et al.* (1971) have shown that increased lipogenesis in response to insulin is partly brought about by activation of pyruvate-dehydrogenase, an enzyme which regulates the generation of acetyl coenzyme A for fatty acid biosynthesis.

They have also shown that this enzyme is activated four- to eight-fold by calcium 10^{-8}–10^{-4}M (Martin *et al.* 1972). We have similarly found that the conversion of glycogen synthetase from the inactive D form to the active I form is promoted by increasing the calcium concentration from 10^{-8}M to 10^{-3}M (Hope-Gill *et al.* 1973, 1974). Calcium ions are also essential for the transport of amino acids and for their incorporation into protein (Cameron & Lejohn 1972).

(4) *Can we simulate insulin's action by altering the intracellular distribution of calcium by other means?* To assess this, we have done some of the standard experiments already described incubating fat cells with drugs known to alter the intracellular calcium distribution and compared these effects with insulin's (Kissebah, Tulloch *et al.* 1974). Local anæsthetics inhibit cellular calcium efflux (from perfused rat liver: Friedman & Rasmussen 1970). Procaine also inhibited adrenaline-induced calcium efflux from rat fat cells, as we shall see. So procaine may well raise cytosol calcium concentration.

Fig 4 shows us the crucial part of a standard experiment as Fig 1, in which procaine reduced both adrenaline-stimulated lipolysis without altering the cAMP level and the state of the fat cell's protein-kinase activation – all as insulin had done. Procaine also induces other insulin-like effects. In fat cells it increases the uptake of glucose or fructose into glycogen, and it is additive in these effects to insulin. Since it has this effect on fructose incorporation as well as on glucose incorporation, this effect cannot have been mediated merely by the specific enhancement of the cell's glucose uptake. And a similar effect from procaine is seen on the incorporation of fructose into lipid.

Nor is this effect unique to procaine – other drugs such as the calcium ionophore (A 23187) and lanthanum, which alter calcium distribution in cells, also have insulin-like antilipolytic action (Fig 5).

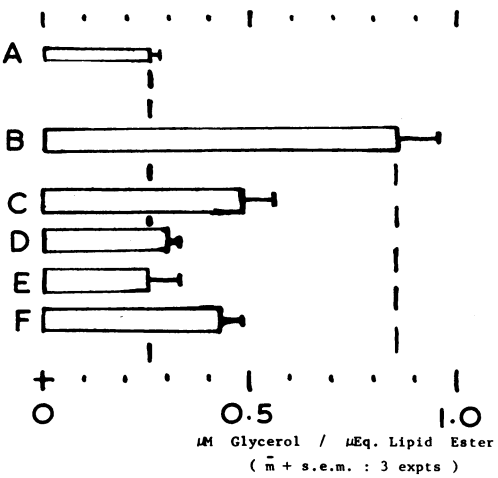

Fig 5 *Effects on lipolysis (glycerol release) of added adrenaline with and without procaine and other drugs, added for 30 min to incubates of rat adipocytes.* A, *control;* B, *with adrenaline 1 μM;* C, *with adrenaline 1 μM + lanthanum 1 mM;* D, *with adrenaline + procaine HCl 5 mM;* E, *with adrenaline 1 μM + calcium ionophore 0.2 μM;* F, *with adrenaline + tolbutamide 1 mM. (Means of three experiments)*

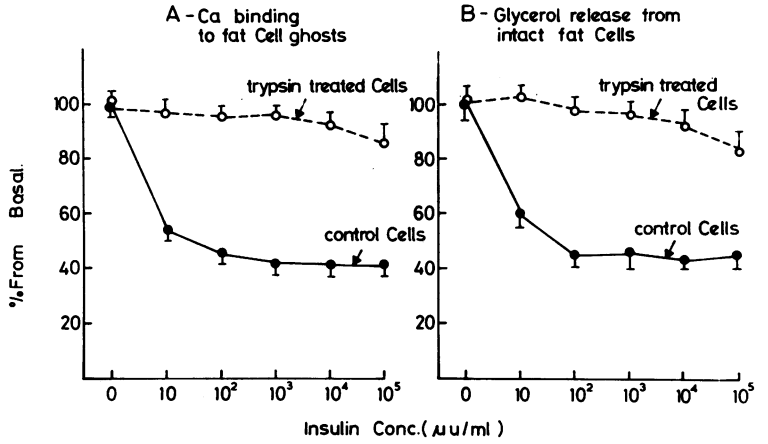

Fig 6 *Retention of calcium binding by ^{45}Ca-prelabelled fat cell ghosts (10 min preincubation in Tris HCl buffer+10 min with added $^{45}CaCl_2$ 0.5 μCi/ml and 5×10^{-6}M, and separation by Millipore filtration) compared with ghosts from similar cells pretreated with trypsin 1 g/l followed by addition of trypsin inhibitor and washing. (Reproduced from Kissebah* et al. *1975, by kind permission)*

(5) *Finally, does insulin really alter cellular calcium in the ways postulated?* It seems that the intracellular calcium can be modulated by a change either (*a*) in calcium distribution between stores and cytoplasm, or (*b*) in the extrusion of calcium from the cell. What, now, are the effects of insulin on these processes?

(*a*) Calcium stores: Insulin seems to increase the mobilization of calcium by decreasing the affinity of calcium binding to membrane preparations. Fig 6 shows the binding of calcium to fat cell ghosts treated with insulin. These preparations serve as a useful model since they retain their insulin responsiveness (Rodbell 1967), which can be deleted by pretreatment with trypsin (Glieman *et al.* 1972). In untrypsinized fat cells, insulin inhibits lipolysis, and in ghosts prepared from the same pool insulin decreased the calcium binding. In trypsinized cells which cannot respond to insulin as regards glycerol release, insulin did not affect ^{45}Ca binding to their ghosts.

(*b*) Calcium efflux: Fat cells were prelabelled with ^{45}Ca and perifused to determine the rate at

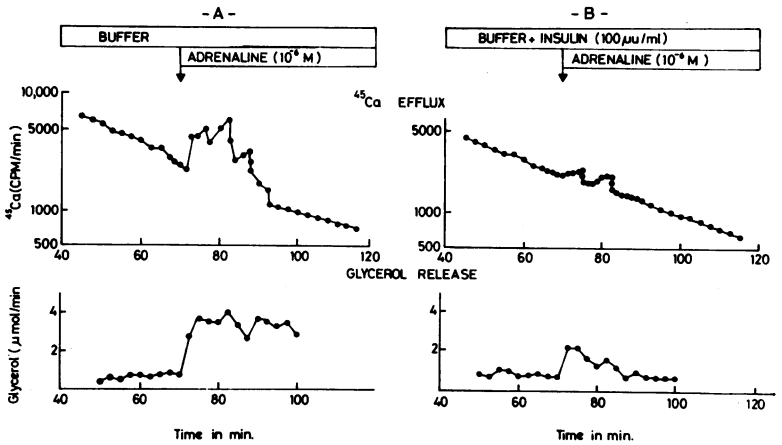

Fig 7A, *'^{45}Ca washout' curve from perifused fat cells prelabelled with ^{45}Ca glycerol release (below)* and B. *'^{45}Ca washout' curve from ^{45}Ca-prelabelled fat cells (above); showing suppression by insulin of lipolysis and also of ^{45}Ca efflux induced by adrenaline. Throughout chamber* A *is perifused with buffer and chamber* B *with buffer+insulin; then at 70 min adrenaline is added to each perifusate (perifusing concentration 10^{-6}*M). *(Reproduced from Kissebah* et al. *1975, by kind permission)*

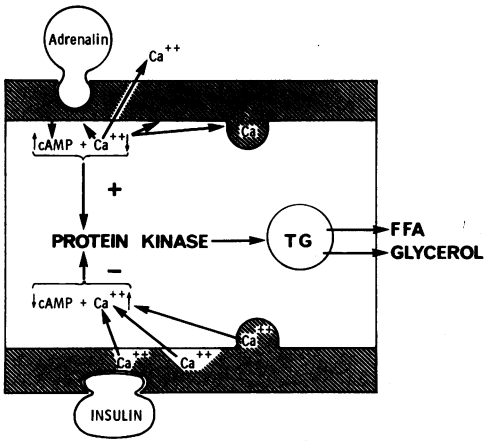

Fig 8 *Proposed scheme for control of lipolysis in fat cells by two second messengers under hormone control*

which ^{45}Ca efflux proceeds. If adrenaline is added at 70 minutes (i.e. during the steady efflux period of Fig 2), a brisk rise in ^{45}Ca efflux occurs which is paralleled by increased glycerol release (Fig 7A). On the other hand, after the cells have been pretreated with insulin the adrenaline-stimulated increase of calcium efflux is almost completely eliminated (Fig 7B). And again the corresponding glycerol release is also almost eliminated. Thus, we see that changes in the cell's distribution of calcium which might correspond with the hypothesis can be induced after adding insulin.

(6) *A mechanism?* Thus, it may be concluded that insulin probably acts on cells by releasing calcium into the cytosol. Wolf (1972) has proposed a model which may define some of the mechanisms involved in insulin's effect on the membrane. The cleavage of adenosine triphosphatase by the cell membrane's calcium adenosine triphosphatase may be possible only when calcium is bound in a specific site in the enzyme. Possibly insulin displaces calcium from these binding sites in the membrane, and thereby inhibits this enzyme, so reducing the calcium efflux and permitting cell uptake of substrates instead, while also raising cytosol calcium.

Conclusion

We believe that the findings reviewed justify the hypothesis outlined on Fig 8 which offers a unitary mechanism for the cellular action of insulin:

(1) On binding to its receptor, insulin alters the conformation of the membrane and so calcium is displaced from high affinity binding sites in the membrane.

(2) This alteration reduces calcium efflux and increases the transport of glucose and other substrates into the cell.

(3) The rise of cytosol calcium promotes dephosphorylation, and so activates or inactivates cellular enzymes corresponding with insulin's known anabolic effects (e.g. inhibiting triglyceride lipase and glycogen phosphorylase and activating glycogen synthetase and pyruvate dehydrogenase).

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