Extracellular calcium modulates insulin's action on enzymes controlling cyclic AMP metabolism in intact hepatocytes

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Absence of physiological concentrations of extracellular Ca^{2+} in the Krebs–Henseleit incubation buffer did not affect the ability of 10 nM glucagon (< 5%) to increase hepatocyte intracellular cyclic AMP concentrations, but severely ablated (by ~ 70%) the ability of 10 nM insulin to decrease these elevated concentrations. Cyclic AMP metabolism is determined by production by adenylate cyclase and degradation by cyclic AMP phosphodiesterase (PDE). In the absence of added extracellular Ca^{2+} (2.5 mM), insulin's ability to activate PDE activity was selectively compromised, showing a failure of insulin to activate two of the three

insulin-stimulated activities, namely the 'dense-vesicle' and peripheral plasma-membrane (PPM) PDEs. In the absence of added Ca^{2+} , insulin's ability to inhibit adenylate cyclase activity in intact hepatocytes was decreased dramatically. Vasopressin and adrenaline (+ propranolol) failed to elicit the activation of either the 'dense-vesicle' or the PPM-PDEs. The presence of physiological concentrations of extracellular Ca^{2+} in the incubation medium is shown to be important for the appropriate generation of insulin's actions on cyclic AMP metabolism.

INTRODUCTION

It is well established that, in hepatocytes, insulin can antagonize the ability of glucagon to increase the intracellular concentration of cyclic AMP (see, e.g., Pilkis et al., 1975; Blackmore et al., 1979; Heyworth and Houslay, 1983). It achieves this by both increasing cyclic AMP phosphodiesterase (PDE) activity (Heyworth and Houslay, 1983) and also by exerting a small inhibitory effect on adenylate cyclase activity (Heyworth and Houslay, 1983). The controls imposed on these systems are complex and subject to distinct time-dependent regulation (Heyworth and Houslay, 1983; Wallace et al., 1984). In hepatocytes, multiple PDE isoforms have their activities regulated by insulin (Houslay and Kilgour, 1990). These include the so-called 'dense-vesicle' enzyme (Loten et al., 1978; Heyworth and Houslay, 1983), which is a type-III PDE (Beavo, 1990), the cognate enzyme of which in adipocytes is activated by insulin through serine phosphorylation (Manganiello et al., 1990). The 'dense-vesicle' PDE can also be activated by glucagon (Loten et al., 1978; Heyworth and Houslay, 1983) through a mechanism which involves its cyclic AMP-dependent phosphorylation (Kilgour et al., 1989). Another key insulin-regulated enzyme is the so-called 'peripheral plasma membrane, (PPM) PDE, which is a type-IV phosphodiesterase (Beavo, 1990), whose activation by insulin in intact cells can be obliterated by pre-exposure of the cells to glucagon (Heyworth et al., 1983). As well as these two species of PDE which can be activated by insulin in intact hepatocytes, there is evidence which suggests the presence of yet another insulin-activated PDE (Heyworth et al., 1984). Although the identity of this species has not been unequivocally made, it is thought that it may reflect the activity of a type-II cyclic GMP-activated PDE (Houslay, 1990).

The presence of extracellular Ca^{2+} at physiological levels is important for the functioning of a variety of hormones which act on hepatocytes (Barritt and Hughes, 1991; Polverino and Barritt, 1988). Indeed, in hepatocytes, extracellular Ca^{2+} has been shown to bind to the insulin receptor and to increase its affinity for

insulin (Williams and Turtle, 1981), to increase the insulin-stimulated phosphorylation of the insulin receptor and other membrane proteins (Plehwe et al., 1983) and to be required for insulin-stimulated oxidation of succinate (Rogers et al., 1991). Extracellular Ca^{2+} at mM levels has also been shown to be essential in allowing for the maximal stimulation by insulin of glucose uptake in adipocytes and soleus muscle (Akhtar and Perry, 1979; Czech, 1980) and for insulin-stimulated DNA synthesis in mammary gland (Zwierchowski et al., 1984).

In this study we assess the requirement for physiological levels of extracellular Ca^{2+} as regards insulin's ability to antagonize glucagon's action in elevating intracellular cyclic AMP levels. We focus on actions exerted at the level of both adenylate cyclase and two defined PDEs.

MATERIALS AND METHODS

[^{32}P]P_i was obtained from Amersham International. Hormones were from Sigma. All other biochemicals were from Boehringer, and other chemicals were of A.R. grade from BDH.

Hepatocyte preparation

Hepatocytes were prepared as previously described (Heyworth and Houslay, 1983) from 220–250 g fed male Sprague–Dawley rats. Cells (10^6 – 10^7 /ml) were preincubated for 20 min at 37 °C in Krebs–Henseleit buffer supplemented with 2.5% BSA and 10 mM glucose. Cells were gassed with O_2/CO_2 (19:1) for 30 s every 5 min. Where stated, the Krebs–Henseleit incubation buffer was also supplemented with 2.5 mM CaCl_2 .

Determination of intracellular cyclic AMP

This was done as described previously (Whetton et al., 1983) where hepatocytes were centrifuged through a layer of bromododecane into a mixture of HClO_4 /sucrose before neutralization

Abbreviations used: PDE, cyclic AMP phosphodiesterase; PIA, N^6 -(phenylisopropyl)adenosine; IBMX, 3-isobutyl-1-methylxanthine; PPM-PDE, type-IV peripheral plasma-membrane PDE.

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and assay of intracellular cyclic AMP by a competition binding procedure.

PDE assay

PDE activity was determined at a final concentration of 100 μM cyclic AMP as substrate by a two-step assay procedure (Thompson and Appleman, 1971) as modified and detailed by us previously (Marchmont and Houslay, 1980). Initial rates were taken from linear time courses.

Assessment of hormonal activation of PDE

Assessment of the activation of the 'dense-vesicle' and PPM PDEs was done as described previously (Heyworth et al., 1983), the Percoll fractionation procedure being used to separate out the various subcellular components of hepatocytes. It was crucial to ensure a good separation of the plasma membranes from the endoplasmic-reticulum fractions, otherwise the significant activity in those fractions obscured the activation of the plasma-membrane enzyme. Such a separation was assured, given an appropriate 'calibration' of each batch of Percoll to give the required separation of marker enzymes (Heyworth et al., 1983). This was done by adjusting the speed of centrifugation to yield the required separation profile (Heyworth et al., 1983).

Determination of free Ca^{2+}

The level of free Ca^{2+} in the hepatocyte incubation mixture which had not been supplemented with Ca^{2+} was determined by the methods described by Smith and Steel (1992). This was done on isolated medium after removal of hepatocytes by centrifugation (Heyworth et al., 1983).

Figure 1 Insulin antagonizes the action of glucagon in elevating intracellular cyclic AMP levels

Data show the time-dependent alteration in intracellular cyclic AMP concentrations elicited by 10 nM glucagon alone (●, ○) and the levels recorded when 10 nM insulin was added some 5 min after the addition of glucagon (□, ■). Experiments were done with cells to which 2.5 mM Ca^{2+} either had (□, ○) or had not (■, ●) been added to the Krebs-Henseleit incubation buffer. At the indicated time points, cells were harvested to stop the reactions for analysis of intracellular cyclic AMP concentrations. The results show a typical experiment of one done with four different hepatocyte preparations. Results are the means of three separate samples taken for determination of intracellular cyclic AMP, with errors expressed as S.D.

Table 1 Modulation by insulin of hepatocyte intracellular cyclic AMP concentrations

Hepatocytes were incubated at 37 °C as described in the Materials and methods section. In some instances, as indicated, 2.5 mM Ca^{2+} was added to the incubation buffer. After incubation for the designated time, cells were rapidly harvested for the determination of intracellular cyclic AMP levels as described in the Materials and methods section. In Section A, cells were pre-treated with 10 nM glucagon for 5 min before challenge, where indicated, with 10 nM insulin. After 5 min challenge with glucagon, cyclic AMP levels attained a maximum and then subsequently decreased (Figure 1). Recorded below is the effect of adding 2.5 mM Ca^{2+} to the incubation buffer on the 'apparent rates of change' produced by insulin on intracellular cyclic AMP concentrations elevated by glucagon. Rates of change in Section A are thus assessed over the apparently linear segments which follow the fall in cyclic AMP accumulation during a period some 5–15 min after glucagon challenge for experiments done with glucagon with or without Ca^{2+} and over 5–12 min for glucagon+insulin without Ca^{2+} and over 5–7 min for glucagon+insulin together with Ca^{2+} . This is reflected by the data shown in Figure 1. This provides an indicator of the relative magnitude of insulin-stimulated PDE activity in achieving enhanced decrease in intracellular cyclic AMP levels. In Section B, cells were preincubated for 15 min with 3-isobutyl-1-methylxanthine (IBMX) to inhibit hepatocyte PDE activity and then challenged with 10 nM glucagon either alone or together with 10 nM insulin. The rates of cyclic AMP accumulation thus reflects adenylate cyclase activity in the absence of any insulin-regulated PDE species. Rates were assessed over an initial 4 min period during which they were linear, as exemplified in the data shown in Figure 3. The changes in the rates of accumulation of cyclic AMP were assessed in cells incubated either with or without 2.5 mM Ca^{2+} added to the incubation buffer. The results shown are the means of three separate experiments done with different hepatocyte cell preparations, with errors expressed as S.D. 'Initial rates' are in pmol of cyclic AMP/min per mg dry wt. of cells, with the insulin-derived increase shown for Section A (glucagon added at 0 min, insulin at 5 min) and decrease for Section B (glucagon and insulin added together). Thus in Section A insulin is seen to enhance the rate at which intracellular cyclic AMP levels fall, whereas in Section B insulin is seen to inhibit the glucagon-stimulated increase. In each experiment, determinations were made at five different time points: n/a, not applicable. † Significant at $P < 0.001$, and * is not significant, for an effect of insulin (Student's t test).

Section	Ca^{2+} and/or insulin	Apparent rate of change in cyclic AMP concentrations	Increase (A) or decrease (B) by insulin
A	+ Ca^{2+} , no insulin	0.8 ± 0.15	n/a
	No. Ca^{2+} , no insulin	0.7 ± 0.1	n/a
	+ Ca^{2+} , + insulin	$3.8 \pm 0.5^\dagger$	3.0
	No Ca^{2+} , + insulin	$1.7 \pm 0.2^\dagger$	1.0
B	+ Ca^{2+} , no insulin	1.2 ± 0.1	n/a
	No Ca^{2+} , no insulin	1.3 ± 0.2	n/a
	+ Ca^{2+} , + insulin	$0.4 \pm 0.1^\dagger$	0.8
	No Ca^{2+} , + insulin	$1.0 \pm 0.1^*$	0.3

RESULTS AND DISCUSSION

A number of very different studies have indicated that the presence of extracellular Ca^{2+} at mM levels may be important for either allowing or optimizing certain actions of insulin (Akhtar and Perry, 1979; Czech, 1980; Williams and Turtle, 1981; Plehwe et al., 1983; Zwierchowski et al., 1984; Rogers et al., 1991). In hepatocytes, insulin effects a well-characterized ability to antagonize the action of glucagon in elevating intracellular cyclic AMP concentrations (see, e.g., Pilkis et al., 1975; Blackmore et al., 1979; Heyworth and Houslay, 1983).

Here we demonstrate that, although failure to include 2.5 mM Ca^{2+} in the Krebs-Henseleit incubation medium did not alter the ability (< 5% difference; $n = 4$ separate experiments using different cell preparations) of 10 nM glucagon to increase intracellular cyclic AMP levels (Figure 1; Blackmore et al., 1979), it markedly attenuated the ability of 10 nM insulin to decrease this glucagon-stimulated rise (Figure 1; Table 1). This is clear from analyses (Table 1) of the ability of 10 nM insulin to augment the rate of decrease in cyclic AMP levels occurring some 5 min after challenge of hepatocytes with 10 nM glucagon. Note,

however, that the lack of adding Ca^{2+} to the extracellular medium did not alter the rate of decrease in intracellular cyclic AMP levels seen in cells challenged with glucagon alone. The potentiating effect of added extracellular Ca^{2+} was limited to modulating the insulin response, which was decreased by some 3-fold (Table 1). Free Ca^{2+} levels were determined in the incubation buffer of hepatocytes to which no Ca^{2+} had been added. In these instances, cells were removed by centrifugation and then free Ca^{2+} levels were determined by using the dye Indo-1 (see the Materials and methods section; Smith and Steele, 1992). When no Ca^{2+} was added to the medium, the free Ca^{2+} levels were 320 ± 15 nM in the incubation mixture which had not been exposed to cells, 330 ± 21 nM in medium incubated with cells for 20 min at 37°C and 350 ± 38 nM in medium incubated with cells for 50 min at 37°C ($n = 3$ separate experiments).

In situations where hepatocytes were challenged with insulin some 5 min after addition of glucagon, it has been shown (Heyworth et al., 1983) that the action of insulin in decreasing cyclic AMP levels was primarily achieved through an increase in PDE activity rather than any marked inhibition of adenylate cyclase. Detailed analysis of this has shown that insulin elicits the activation of two distinct membrane-bound PDEs: the 'dense-vesicle' (Loten et al., 1978; Heyworth and Houslay, 1983; Heyworth et al., 1983) and PPM enzymes (Heyworth and Houslay, 1983; Heyworth et al., 1983). Treatment of intact hepatocytes gives rise to the stable activation of these two enzymes, which can be assessed upon cell harvesting, subcellular fractionation and membrane isolation (Houslay and Kilgour, 1990; Heyworth et al., 1983). There is, however, strong evidence to suggest that a further, as yet unidentified, PDE is also activated by insulin in intact hepatocytes, although the postulated increase in activity of this species does not appear to survive subcellular fractionation (Heyworth et al., 1984; Houslay, 1990) and may be due to the effect of a soluble activator (Pyne and Houslay, 1988). This unidentified PDE and the 'dense-vesicle' PDE provide the main species through which insulin decreases intracellular cyclic AMP levels raised by glucagon. The PPM-PDE acts primarily to alter basal levels, except in the presence of adenosine, which prevents glucagon blocking insulin's activation of this enzyme (Houslay, 1990).

In order to assess directly the ability of insulin to increase hepatocyte PDE activities, we challenged intact cells either with 10 nM glucagon alone for 10 min or with 10 nM insulin added some 5 min after glucagon, and then rapidly separated out subcellular membranes in order to quantify changes in the membrane PDE activities. Doing this, we were able to show that insulin was unable to elicit activation of either the 'dense-vesicle' PDE or the PPM-PDE when 2.5 mM Ca^{2+} had not been added to the cell incubation medium (Table 1). Loss of activation of the 'dense-vesicle' PDE is consistent with the much diminished rate at which insulin elicits a decrease in hepatocyte cyclic AMP levels (Figure 1; Table 1). Nevertheless, even when Ca^{2+} was not added to the cell incubations insulin was still able to effect a decrease in intracellular cyclic AMP levels, albeit a much smaller one (Figure 1; Table 1). It would seem that, although the presence of extracellular levels of 2.5 mM Ca^{2+} are necessary for insulin to activate the 'dense-vesicle' PDE, they are not required for activation of the 'unidentified' PDE which contributes to the enhanced rate of degradation of cyclic AMP elicited by insulin. It is then, presumably, the insulin-stimulated increase in activity of this enzyme which is responsible for the residual ability of insulin to decrease cyclic AMP concentrations under these circumstances. In this regard it is of interest that, whereas Claus and Pilkis (1976) showed that mM levels of extracellular Ca^{2+} were necessary for insulin to decrease intracellular cyclic AMP

Table 2 Activation by insulin of hepatocyte PDEs

Hepatocytes were incubated at 37°C as described in the Materials and methods section with, where indicated, 2.5 mM Ca^{2+} present in the incubation buffer during both the 20 min preincubation and the experimental analysis (challenge with hormone). After incubation for the designated time, cells were rapidly cooled by adding ice-cold stopping buffer. They were then harvested, pressurized, homogenized and subsequently fractionated on a Percoll gradient in order to resolve the PPM and 'dense-vesicle' PDE activities (Heyworth et al., 1983). nd, not determined. The results shown are the means of three separate experiments done with different hepatocyte cell preparations, with errors expressed as S.D. Comparing data obtained from experiments done in the presence and absence of 2.5 mM Ca^{2+} added to the hepatocyte incubation mixture, * not significant and † significant at $P < 0.001$. Analysing whether the presence of PIA affected the changes in PDE activity achieved by the exposure of hepatocytes to glucagon before challenge with insulin, ‡ not significant and § significant at $P < 0.001$ (Student's t test). Analysing enzyme activation by adrenaline (+propranolol) and vasopressin, || not significant. The lack of added extracellular Ca^{2+} did not initiate cell breakage over the assay period, as indicated by Trypan Blue exclusion or any alterations in intracellular ATP levels (see the text). Data show the relative PDE activity in the fractions expressed as a percentage of those basal levels found in cells which had not been challenged with these ligands (100%).

	'Dense-vesicle' PDE		PPM-PDE	
	Plus 2.5 mM Ca^{2+}	No Ca^{2+}	Plus 2.5 mM Ca^{2+}	No Ca^{2+}
10 nM insulin (5 min)	131 ± 7	96 ± 4†	185 ± 9	88 ± 13†
10 nM glucagon (5 min)	156 ± 12	168 ± 6*	97 ± 7	101 ± 10*
Glucagon (5 min), then insulin (5 min)	242 ± 15	162 ± 17†	101 ± 4	93 ± 4*
100 nM PIA (1 min) + glucagon (5 min), then insulin (5 min)	238 ± 11‡	166 ± 12‡	172 ± 10§	106 ± 8†
100 nM PIA (11 min)	110 ± 8	105 ± 4*	108 ± 6	103 ± 8*
10 nM vasopressin (5 min)	103 ± 4	nd	101 ± 5	nd
100 µM adrenaline + 100 µM propranolol (5 min)	117 ± 9	nd	104 ± 6	nd

concentrations which had been elevated by glucagon, Blackmore et al. (1979) failed to observe such a clear-cut effect. In neither study was any attempt made to attribute or identify any perceived changes to effects exerted at the level of either cyclic AMP production of adenylate cyclase or degradation by specific isoforms of PDE. However, as insulin's ability to decrease hepatocyte cyclic AMP levels, under conditions of glucagon challenge, depends on insulin increasing the activities of at least two PDE isoforms, and the activation of only one of these appears to be dependent on the addition of 2.5 mM Ca^{2+} , then it is possible that strain-dependent differences in the levels of expression of such PDE isoforms could give rise to cells with very different dependencies of the action of insulin, in lowering cyclic AMP levels, on added extracellular Ca^{2+} . In this regard we, like Claus and Pilkis (1976), used Sprague-Dawley rats for our source of hepatocytes.

This model system shows that physiological levels of extracellular Ca^{2+} have selective effects on insulin-activated PDE activities involved in cyclic AMP metabolism. Selective effects of such levels of extracellular Ca^{2+} also extends to the hormonal control of the 'dense-vesicle' PDE, as it was only the action of insulin, and not that of glucagon, which was abrogated in cells where 2.5 mM Ca^{2+} was omitted from the incubation mixture (Table 2). Thus glucagon's stimulatory effect on either adenylate cyclase or the 'dense-vesicle' PDE was not affected by the failure to add 2.5 mM Ca^{2+} to the hepatocyte incubation buffer. Although activation of the 'dense-vesicle' enzyme has been shown to be abrogated if intracellular ATP levels fall markedly (Boyes et al., 1981; Heyworth et al., 1984), this cannot account

Figure 2 Action of PPM-PDE in lowering intracellular cyclic AMP levels

In these experiments hepatocytes were treated for 5 min with 10 nM glucagon before addition of 10 nM insulin and subsequent taking of samples for determination of intracellular cyclic AMP concentrations. Data show the time-dependent alteration in intracellular cyclic AMP concentrations elicited when 100 nM PIA either was (○, ●) or was not (□, ■) added to the cells 1 min before addition of 10 nM glucagon. Experiments were done with cells to which 2.5 mM Ca^{2+} either had (●, ■) or had not (○, □) been added to the Krebs-Henseleit incubation buffer. The results show a typical experiment of one done with three different hepatocyte preparations. Results are the means of three separate samples taken for the determination of intracellular cyclic AMP, with errors expressed as S.D.

Figure 3 Inhibition by insulin of adenylate cyclase in intact hepatocytes

Data show the time-dependent alteration in intracellular cyclic AMP concentrations elicited by 10 nM glucagon in the absence (●, ○) or presence (■, □) of 10 nM insulin for hepatocytes which had been preincubated for 15 min with 1 mM IBMX before challenge with hormones. Experiments were done with cells to which 2.5 mM Ca^{2+} either had (□, ○) or had not (■, ●) been added to the Krebs-Henseleit incubation buffer. At the indicated time points, cells were harvested to stop the reactions for analysis of intracellular cyclic AMP concentrations. The results show a typical experiment of one done with four different hepatocyte preparations. Results are the means of three separate samples taken for the determination of intracellular cyclic AMP, with errors expressed as S.D.

for the decreased action of insulin here, as no changes in ATP levels occurred when 2.5 mM Ca^{2+} was omitted from the incubation mixture. Thus intracellular ATP levels were 10.4 ± 1.1 nmol of ATP/mg dry wt. of cells in the presence, and 10.9 ± 1.2 nmol of ATP/mg dry wt. of cells in the absence, of added 2.5 mM Ca^{2+} . Furthermore, the effect of removal of Ca^{2+} from the incubation buffer was reversible, as the addition of 2.5 mM Ca^{2+} to cells, after incubation for either 10 or 20 min without it, restored (> 96%) the ability of insulin to activate

these enzymes (results not shown). Thus the effect of added extracellular Ca^{2+} does not appear to have a protracted time-dependent action. Furthermore, such effects of extracellular Ca^{2+} on insulin's action here could be abrogated (> 96%) if, after incubation of cells for 20 min with 2.5 mM Ca^{2+} they were washed, by centrifugation, in medium without added Ca^{2+} and then challenged with insulin. The subsequent re-addition of 2.5 mM Ca^{2+} to the incubation medium would restore the response (results not shown).

Exposure of hepatocytes to glucagon before challenge with insulin has been shown to obliterate the ability of insulin to activate the PPM-PDE (Heyworth et al., 1983; Table 2). However, this inhibitory action of glucagon can be negated by adding either adenosine or the non-metabolizable adenosine analogue N^6 -(phenylisopropyl)adenosine (PIA) (Wallace et al., 1984; Table 2). Taking advantage of this, it is possible to gauge (Figure 2) the ability of the insulin-stimulated PPM-PDE to decrease intracellular cyclic AMP concentrations in intact hepatocytes by monitoring the enhanced decrease in cyclic AMP levels that insulin caused in the presence of PIA (Heyworth et al., 1984; Houslay, 1990). Consistent with the inability of insulin to activate the PPM-PDE in the absence of added extracellular Ca^{2+} , as assessed by direct analysis (Table 2), we observed that the ability of PIA to enhance the lowering of intracellular cyclic AMP concentrations by insulin was markedly decreased (Figure 2) if Ca^{2+} was not added to the incubation medium.

We have shown (Heyworth and Houslay, 1983; Heyworth et al., 1983, 1986) that, although insulin can elicit a small inhibition of adenylate cyclase in intact hepatocytes when added together with glucagon, such an effect is lost in parallel to the onset of glucagon desensitization. It thus plays a marginal role when insulin is added some 5 min after the addition of glucagon (Heyworth et al., 1983). Nevertheless, it is possible to gauge the ability of insulin to inhibit adenylate cyclase in intact hepatocytes by adding insulin and glucagon together and then analysing rates of hepatocyte intracellular cyclic AMP accumulation while in the presence of the non-specific PDE inhibitor IBMX (Heyworth and Houslay, 1983; Heyworth et al., 1983, 1986). Doing this we see that, in the absence of 2.5 mM Ca^{2+} , insulin's ability to inhibit adenylate cyclase was dramatically attenuated (Figure 3; Table 1).

The molecular basis of the action of extracellular Ca^{2+} in these events remains to be defined. There is no evidence to suggest that insulin causes entry of Ca^{2+} into hepatocytes and, indeed, such an action would be expected to stimulate metabolic processes that insulin in fact antagonizes (see Denton et al., 1981). Furthermore, examination of the action of hormones such as vasopressin and adrenaline (α_1 -adrenoceptor action), which elicit an increase in intracellular Ca^{2+} (Hems and Whitton, 1980; Exton, 1980), shows that they are incapable of activating either of these PDEs (Table 2). Thus, unless insulin allows a Ca^{2+} flux into a discrete compartment within these cells, it would seem likely that the extracellular Ca^{2+} is exerting its action at the cell surface itself. In this regard it is of interest that extracellular Ca^{2+} has been shown to alter the binding of insulin to its receptor, and also receptor autophosphorylation (Williams and Turtle, 1981; Plehwe et al., 1983). Furthermore, extracellular Ca^{2+} has been shown to be important for endocytotic processes (Baker and Knight, 1981) and, in particular, the receptor-mediated endocytosis of the epidermal-growth-factor receptor (Carpenter and Cohen, 1979; Tupper and Bodine, 1983), a tyrosine kinase which is cognate to the insulin receptor (Houslay and Siddle, 1989). In this regard, endocytosis has been shown to be an important function which can be attributed to the insulin receptor (for review, see Houslay and Siddle, 1989) and also for insulin's

stimulation of glucose transport (Lienhard, 1983), a process shown to be influenced by extracellular Ca^{2+} (Akhtar and Perry, 1979; Czech, 1980). Thus consideration of extracellular Ca^{2+} levels would appear to be of some importance in the characterization of the action of insulin in intact cells and also, perhaps, in isolated membrane systems where insulin actions have often been difficult to characterize and where Ca^{2+} concentrations are also usually vanishingly low.

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