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Cytosolic free Ca^{2+} in insulin secreting cells and its regulation by isolated organelles

by M. Prentki and C.B. Wollheim

Institut de biochimie clinique, University of Geneva, Sentier de la Roseraie, CH-1211 Geneva 4 (Switzerland)

Summary. The role of Ca^{2+} in secretagogue-induced insulin release is documented not only by the measurements of ^{45}Ca fluxes in pancreatic islets, but also, by direct monitoring of cytosolic free Ca^{2+}, $[Ca^{2+}]_i$. As demonstrated, using the fluorescent indicator quin 2, glyceraldehyde, carbamylcholine and alanine raise $[Ca^{2+}]_i$ in the insulin secreting cell line RINm5F, whereas glucose has a similar effect in pancreatic islet cells. The regulation of cellular Ca^{2+} homeostasis by organelles from a rat insulinoma, was investigated with a Ca^{2+} selective electrode. The results suggest that both the endoplasmic reticulum and the mitochondria participate in this regulation, albeit at different Ca^{2+} concentrations. By contrast, the secretory granules do not appear to be involved in the short-term regulation of $[Ca^{2+}]_i$. Evidence is presented that inositol 1,4,5-trisphosphate, which is shown to mobilize Ca^{2+} from the endoplasmic reticulum, is acting as an intracellular mediator in the stimulation of insulin release.
Key words. Pancreatic B-cell; insulin secretion; cytosolic free Ca^{2+}.

Introduction

Ca^{2+} plays an important role in cell activation in general[11,25] and in stimulus-secretion coupling in the B-cell in particular[30,60]. The large body of evidence emphasizing the importance of Ca^{2+} in the regulation of insulin release from the pancreatic B-cell was reviewed in depth previously[100]. Although overwhelming, the evidence remained circumstantial that cytosolic free Ca^{2+}

increases during stimulation of insulin release since direct monitoring of changes in cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, was not possible. This has now become feasible by the use of the intracellularly trapped fluorescent Ca^{2+} indicator, quin 2[97]. The availability of insulin secreting cell lines and insulinomas with maintained sensitivity to a number of insulin secretagogues, has facilitated the application of the quin 2 method.

To gain insight into the regulation of cytosolic Ca^{2+}, it is also necessary to study Ca^{2+} transport by various cellular organelles. The isolation of such organelles is also greatly facilitated by the use of insulinomas, where the amount of tissue is not as limited as when isolated pancreatic islets are employed. The present review describes the results obtained with quin 2 in intact cells and with Ca^{2+} selective electrodes applied to isolated organelles for assessment of their Ca^{2+} transport characteristics. The application of these techniques for the direct measurement of Ca^{2+} handling has, in several instances, altered the interpretation of earlier findings based on $^{45}Ca^{2+}$ flux studies.

Ca^{2+} handling by the B-cell

Before entering into the description of our recent experiments, a current model of Ca^{2+} handling in the B-cell may serve as background information (fig. 1). To a minor extent, Ca^{2+} enters the cell by diffusion along the inwardly directed electrochemical gradient. The maintenance of this ionic gradient, which amounts to approximately 10,000 in the resting state, depends on Ca^{2+} extruding mechanisms at the plasma membrane and Ca^{2+} sequestration by intracellular organelles.

At the plasma membrane two processes are involved: a Ca^{2+} pump, which derives its energy from ATP hydrolysis, and a Na^+/Ca^{2+} countertransport driven by the inward Na^+ gradient (for reviews, see Blaustein and Nelson[10] and Schatzmann[83]). The former enzyme, which was demonstrated in subcellular fractions of rat islets enriched in plasma membrane, has a high affinity for Ca^{2+}, is activated by Ca^{2+} calmodulin and is Mg^{2+} dependent[53,71]. Na^+/Ca^{2+} countertransport was studied by measuring $^{45}Ca^{2+}$ efflux from isotope preloaded islets. When Na^+ is removed from the perifusing medium and replaced by choline and sucrose[42,48] or K^+[39], a decrease of $^{45}Ca^{2+}$ efflux is observed. Under conditions

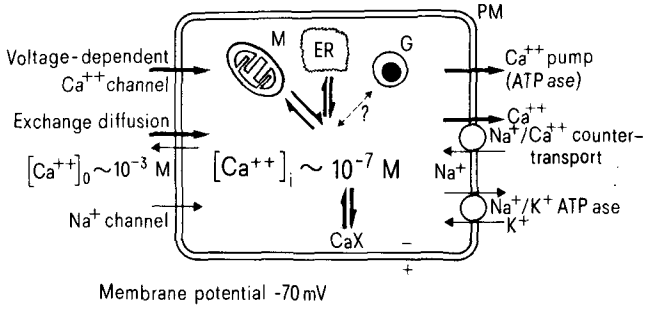

Figure 1. Ca^{2+} handling by the B-cell. The abbreviations are: M, mitochondria; ER, endoplasmic reticulum; G, insulin-containing secretory granules; PM, plasma membrane; CaX, calcium buffering by cytoplasmic components including calmodulin and small molecules. In addition, calcium is also bound to membrane phospholipids.

where $^{45}Ca^{2+}$ efflux can be equated with $^{40}Ca^{2+}$ efflux (islets loaded to isotopic equilibrium and perifused with EGTA-containing Ca^{2+}-free medium) Na^+/Ca^{2+} countertransport seems to contribute to 30% of basal Ca^{2+} efflux[48]. However, the relative proportion of Na^+ dependent Ca^{2+} efflux may be greater in the presence of external Ca^{2+} and under stimulated conditions when $[Ca^{2+}]_i$ is higher than in the absence of external Ca^{2+}. It should be noted that the Na^+/Ca^{2+} countertransport, albeit not directly energy dependent, relies on the preservation of the inwardly directed Na^+ gradient, which, in turn, is maintained by the activity of the Na^+ pump (Na^+/K^+ ATPase). Inhibition of the Na^+ pump by ouabain which increases cellular $[Na^+]$ also decreased $^{45}Ca^{2+}$ efflux at early time points after its addition[90].

Various cellular organelles such as mitochondria, endoplasmic reticulum, and perhaps secretory granules participate in the regulation of cytosolic Ca^{2+} homeostasis. This will be discussed in a later section. Many cytoplasmic proteins bind Ca^{2+} with a high affinity. This bound calcium has been estimated in other tissues to represent more than 99% of the total calcium present in the cytosol[11].

Stimulators of insulin release may raise cytosolic $[Ca^{2+}]$ by four different means: 1. an increased permeability of the plasma membrane to Ca^{2+} by the opening of voltage-dependent or receptor-activated Ca^{2+} channels, resulting in the passive entry of Ca^{2+} along its electrochemical gradient; 2. by inhibition of Ca^{2+} efflux across the plasma membrane; 3. by mobilization of Ca^{2+} from intracellular stores; and 4. by inhibition of Ca^{2+} sequestration by the stores. The existence of voltage-dependent Ca^{2+} channels has been demonstrated by both electrophysiological[4,66] and $^{45}Ca^{2+}$ flux studies[98,100,101]. Thus, glucose, glyceraldehyde and high K^+ concentrations depolarize the B-cells[4,64,66] and increase $^{45}Ca^{2+}$ influx[60,98,100,101], which can be attenuated by channel blockers such as verapamil[60,98] and Co^{2+}[40,101]. That depolarization with high K^+ raises $[Ca^{2+}]_i$ has recently been demonstrated for the insulin secreting cell line RINm5F[81,102] and isolated mixed islet cells[82].

Effects of glucose on $^{45}Ca^{2+}$ fluxes

At physiological Ca^{2+} concentrations (1 mM), the biphasic insulin release elicited by glucose is accompanied by a biphasic increase in $^{45}Ca^{2+}$ efflux from islets preloaded with the isotope (fig. 2A)[100]. However, in the presence of extracellular Ca^{2+}, the specific radioactivity of the intracellular $^{45}Ca^{2+}$ decreases continuously, in particular under conditions of stimulated Ca^{2+} influx. Therefore, strict interpretation of $^{45}Ca^{2+}$ efflux is only possible when islets have been preloaded with the isotope (to attain isotopic equilibrium) followed by perifusion in the absence of extracellular Ca^{2+}[100]. Under these conditions glucose causes only a decrease of $^{45}Ca^{2+}$ efflux, which can now be equated to a true decrease of $^{40}Ca^{2+}$ efflux (fig. 2D)[33,48,100]. Despite the inhibition of Ca^{2+} efflux, glucose is unable to stimulate insulin release in the absence of extracellular Ca^{2+}[100]. In this situation glucose is probably unable to raise $[Ca^{2+}]_i$ in the B-cells above a critical threshold as the cells are gradually depleted of Ca^{2+}[48].

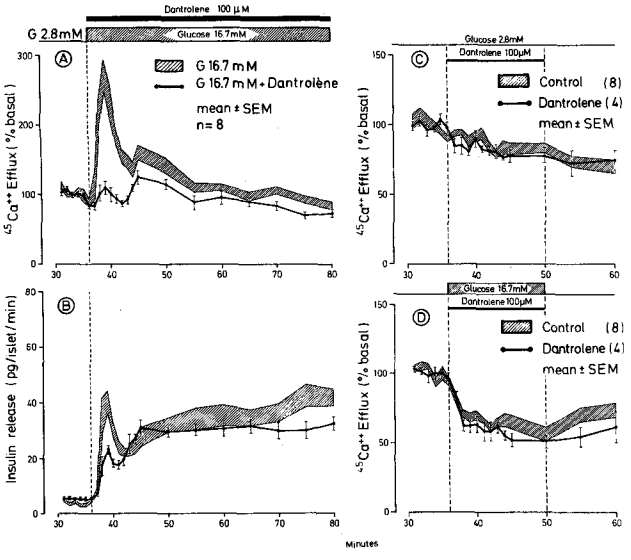

Figure 2. Effect of glucose and dantrolene on ^{45}Ca efflux and insulin release. *A* and *B* Perifusion in the presence of 1 mM extracellular Ca^{2+}. *C* and *D* Perifusion in Ca^{2+}-free medium containing 0.1 mM EGTA. For details, see Janjic et al.[47].

It has been suggested that the increase of $^{45}Ca^{2+}$ efflux evoked by glucose in Ca^{2+}-containing medium reflects, at least in part, Ca^{2+} mobilization from cellular stores[47, 55, 100]. This interpretation is favored by the finding that dantrolene, a substance that inhibits Ca^{2+} release from the sarcoplasmic reticulum[23, 24] abolishes glucose-induced $^{45}Ca^{2+}$ efflux (fig. 2A)[47]. Under these conditions, only the first phase of insulin release is attenuated (fig. 2B). This finding is consistent with the proposed dependency on Ca^{2+} mobilization of the first phase of insulin release[100]. Other studies have suggested that the stimulated $^{45}Ca^{2+}$ efflux is due to a $^{40}Ca^{2+}$–$^{45}Ca^{2+}$ exchange[41, 51] occurring when Ca^{2+} influx is enhanced during glucose stimulation[37, 60, 100]. This explanation seems unlikely, since dantrolene only slightly affects glucose-stimulated $^{45}Ca^{2+}$ uptake but abolishes $^{45}Ca^{2+}$ efflux[47]. Moreover, verapamil abolishes glucose-stimulated $^{45}Ca^{2+}$ uptake but only causes a small decrease in $^{45}Ca^{2+}$ efflux[98]. It is also possible that the small decrease in $^{45}Ca^{2+}$ efflux. It is also possible that the failure of glucose to stimulate ^{45}Ca efflux in Ca^{2+}-free medium could be due to a reduced production of a Ca^{2+} mobilizing intermediate, e.g. inositol 1, 4, 5-trisphosphate under this experimental condition (see last section). It should be added that the islets display a net gain in total calcium during glucose-stimulation[31, 50, 60, 79, 91] (due to the imbalance between Ca^{2+} uptake and efflux) although in some reports[1, 104] this could not be observed.

The mechanism underlying the reduction in Ca^{2+} efflux (seen in Ca^{2+}-free medium) is not clear. Two interpretations have been put forward: the first localizes the action of glucose and other nutrient stimuli to the plasma membrane[42, 43, 48]; the second suggests that these agents enhance Ca^{2+} sequestration by intracellular organelles[38, 39]. As the removal of external Na^+ exerts the same effect as glucose on $^{45}Ca^{2+}$ efflux (in Ca^{2+}-free medium) and since there is no additivity of the effects, it was proposed that glucose acts by interfering with the plasma membrane Na^+/Ca^{2+} countertransport[42, 90]. This view has recently been challenged since glucose was shown to decrease $^{45}Ca^{2+}$ efflux in the absence of Na^+, provided the ion was replaced by K^+[39]. Alternatively, it has been proposed that acidification of the cytosol mediates the reduction of Ca^{2+} efflux evoked by glucose[17, 43]. Thus, manipulations that lower cytosolic pH, mimick qualitatively the effect of glucose on $^{45}Ca^{2+}$ efflux[17], and on electrical activity[70]. This view is strengthened by the recent finding that glycoraldehyde and glucose lower cytosolic pH in B-cells as assessed directly by the intracellularly trapped fluorescent probe, bis-carboxy-ethylcarboxyfluorescein[103]. By contrast, previous reports employing substances whose distribution is not restricted to the cytosol, showed an alkalinization of the islet cells following glucose exposure[22, 56].

It is clear from the data discussed so far that the results obtained with the $^{45}Ca^{2+}$ method are often difficult to interpret, since changes in ^{45}Ca fluxes do not necessarily reflect $^{40}Ca^{2+}$ movements[12, 100]. This is certainly the reason for some of the conflicting views found in the literature. However, new methods allowing the direct monitoring of the free Ca^{2+} concentration have been developed. The results obtained with these techniques will now be described.

Measurement of $[Ca^{2+}]_i$ in insulin secreting cells

With the introduction of the quin-2 method[97], it has become possible to monitor continuously the cytosolic free Ca^{2+} concentration in various cell types[18, 69, 72, 85]. The usual application of this technique requires large amounts of cells. To this end, we have used as a model of pancreatic B-cells the insulin-secreting cell line RINm5F. Insulin release from this cell line is elicited by a variety of secretagogues with the notable exception of glucose[32, 35, 73]. The lack of glucose responsiveness may be due to the deficiency in the high K_m phosphorylating enzyme, glucokinase-like[35]. Indeed this enzyme appears to catalyze the rate-limiting step of glucose metabolism by the B-cells and consequently of glucose-induced insulin release by the B-cells[65]. As nutrient secretagogues, we used therefore the triose D-glyceraldehyde and the amino acid L-alanine, both of which also stimulate insulin secretion from normal pancreatic B-cells[55, 59, 91].

The resting $[Ca^{2+}]_i$ in suspensions of RINm5F cells was found to be 105 ± 6 nM[102], a value similar to those reported for other cell types [18, 69, 72, 85]. As can be seen in figure 3C, D-glyceraldehyde (10 mM) elevated $[Ca^{2+}]_i$ about 3-fold within 2 min. The pattern of the Ca^{2+} rise was similar to that of the change in membrane potential, as assessed with the fluorescent probe bisoxonol[102] (fig. 3A). L-alanine (10 mM) also depolarized the cells (fig. 3B) and elevated $[Ca^{2+}]_i$ (fig. 3D). To know whether the rise in $[Ca^{2+}]_i$ following alanine addition was due to the opening of voltage-dependent Ca^{2+} channels, the channel blocker verapamil was used. Verapamil rapidly returned $[Ca^{2+}]_i$ to the resting level (fig. 3D). Verapamil was also used to evaluate the relative importance of Ca^{2+} influx (through Ca^{2+} channels) and Ca^{2+} mobilization in the increase of $[Ca^{2+}]_i$ and insulin release. Under various experimental conditions, a correlation between $[Ca^{2+}]_i$ and insulin release was observed[102] (fig. 4). When

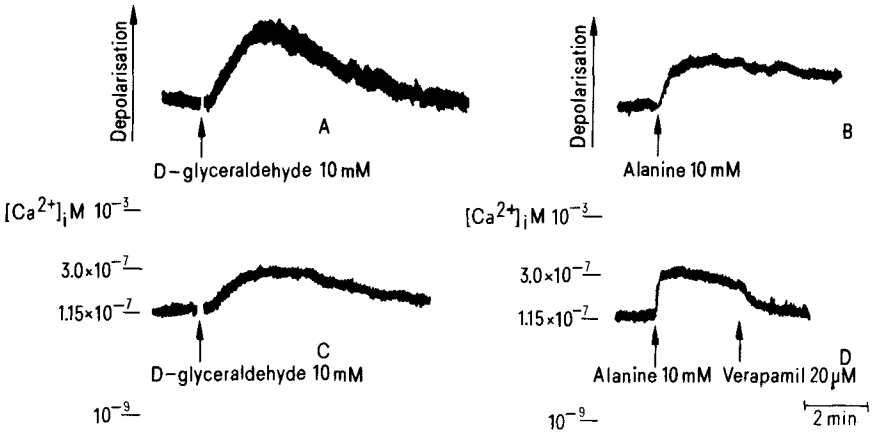

Figure 3. Effect of gylceraldehyde and alanine on membrane potential (A and B) and $[Ca^{2+}]_i$ (C and D). In suspension of RINm5F cells, membrane potential was monitored following the addition of the fluorescent probe bisoxonol at a final concentration of 100 nM. $[Ca^{2+}]_i$ was calculated from changes in quin-2 fluorescence. For details, see Wollheim and Pozzan[102].

cells were exposed to verapamil prior to the addition of glyceraldehyde or alanine, $[Ca^{2+}]_i$ was reduced by 50% and 80%, respectively. The decreases in insulin release paralleled the changes in $[Ca^{2+}]_i$ (fig. 4). It should be noted that, as for K^+ depolarization[81, 102], the alanine induced rise in $[Ca^{2+}]_i$ can be explained entirely by Ca^{2+} influx through voltage-dependent Ca^{2+} channels. Glyceraldehyde, on the other hand, appears to raise $[Ca^{2+}]_i$ not only be promoting Ca^{2+} influx, but also by Ca^{2+} mobilization from internal stores[55, 100, 102]. This conclusion is based on the finding that even when maximal concentrations of verapamil or diltiazem (another channel blocker) are used, a residual elevation in $[Ca^{2+}]_i$ remains[102] (fig. 4). This substantiates the hypothesis formulated previously[98, 100] which suggested that glucose uses both intra- and extracellular Ca^{2+} to raise $[Ca^{2+}]_i$.

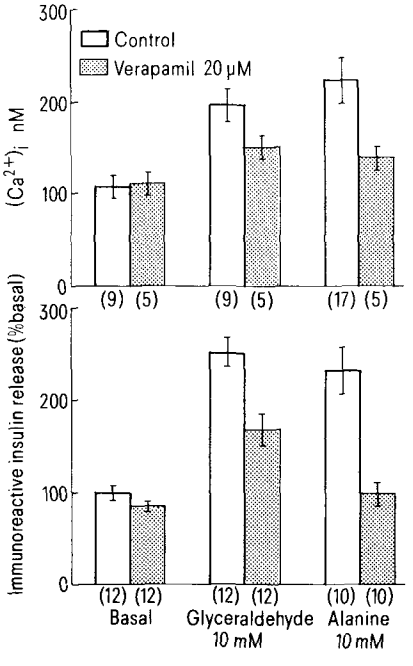

Figure 4. Effect of verapamil on the rise in $[Ca^{2+}]_i$ and insulin release induced by glyceraldehyde and alanine. Verapamil was added 5 min prior to the secretagogues. For the stimulating conditions, peak $[Ca^{2+}]_i$ is shown. Insulin release was measured over 10 min. The values represent mean ± SE of the number of observations indicated in the parentheses.

However, Ca^{2+} mobilization could not so far be demonstrated directly since, in Ca^{2+}-free medium, glyceraldehyde did not raise $[Ca^{2+}]_i$. By contrast, under the same conditions, carbamylcholine did elevate $[Ca^{2+}]_i$[75]. This may be due to a different sensitivity towards EGTA and/or extracellular Ca^{2+} of the process leading to polyphosphoinositide breakdown that is involved in Ca^{2+} mobilization (see below).

To ensure that glyceraldehyde tested in the cell line reproduces the effect of glucose in the B-cell, experiments were carried out using monolayers of adult rat pancreatic islet cells. Insulin release from this preparation was increased by 430 ± 35% (n = 10), when glucose was raised from 2.8 to 16.7 mM (30-min incubation). In these rat islet cells, glucose was found to elevate maximally $[Ca^{2+}]_i$ within 1 min (fig. 5) with the same pattern as that observed with glyceraldehyde in RINm5F cells (fig. 3). Similar findings have recently been reported in mouse pancreatic islet cells in suspension[82].

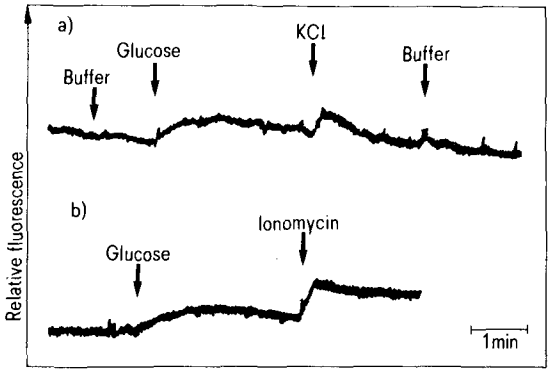

Figure 5. Effect of glucose on quin-2 fluorescence ($[Ca^{2+}]_i$) in monolayers of pancreatic islets. Isolated islet cells were obtained from adult rats as previously described[34]. Cells were plated on glass slides and cultured in RPMI 1640 medium for 4 days. After washing, the attached cells were exposed to 50 µM quin 2 acetoxymethyl-ester for 30 min. the cells were washed and placed in a fluorimeter, and intracellular fluorescence was recorded as described elsewhere[102]. The glucose concentration of the Krebs-Ringer bicarbonate Hepes buffer was 2.8 mM, and was raised to 16.7 mM where indicated (glucose). KCl denotes the change in ion concentration from 6 to 30 mM; ionomycin was used at a final concentration of 1 µM (S. Ullrich, T. Pozzan and C. B. Wollheim, unpublished observations).

Ca^{2+} transport by isolated organelles

In most cell types, the cytosolic Ca^{2+} concentration is regulated by transport systems located not only in the plasma membrane but also in membranes of mitochondria, endoplasmic reticulum, and perhaps secretory granules[11]. In the endocrine pancreas, few studies have dealt with the regulation of Ca^{2+} handling by the various organelles. Subcellular fractionation studies have been hampered by the extremely small quantity of tissue available from pancreatic islets.

In the literature, two approaches have been used in $^{45}Ca^{2+}$ studies with isolated fractions from pancreatic islets. The first involved the labeling with $^{45}Ca^{2+}$ of intact islets followed by secretagogue stimulation and subcellular fractionation. In these experiments, glucose-stimulated pancreatic islets exhibited an increased ^{45}Ca content in all subcellular compartments (mitochondria, microsomes, secretory granules, nuclei) subsequently isolated[2,3,13,52]. It should be emphasized that such results are expected since glucose increases ^{45}Ca uptake by the islets; consequently enhanced Ca^{2+} turnover and ^{45}Ca-^{40}Ca exchange must occur. Furthermore, it is not possible to obviate the artefacts of Ca^{2+} redistribution during organelle isolation. Thus, modifications in the ^{45}Ca content of isolated organelles are not conclusive. Indeed, when the total calcium content of these fractions was measured, the results did not reproduce the changes observed with $^{45}Ca^{2+}$[2].

The second approach concerned ^{45}Ca uptake studies by fractions enriched in subcellular organelles. ATP-dependent $^{45}Ca^{2+}$ uptake has been demonstrated in total islet homogenates[45], and in preparations enriched in mitochondria[93] and microsomes[88]. Active calcium transport by a fraction enriched in the endoplasmic reticulum was shown to be Mg ATP dependent, stimulated by K^+ and had a K_m for Ca^{2+} of 1.5 μM[19]. Neiter calmodulin nor cAMP affected this Ca^{2+} transporting activity[20]. In less well characterized microsomal fractions, the addition of cAMP or theophylline inhibited ^{45}Ca transport[88]. In isolated mitochondria, 3-isobutyl-1-methylxanthine (a drug related to theophylline) as well as the glucose metabolite, phosphoenolpyruvate, were shown to decrease the accumulation of $^{45}Ca^{2+}$[93]. From this observation it was suggested that these molecules might influence exocytosis through a direct effect on mitochondrial Ca^{2+} transport[93]. As phosphoenolpyruvate has been shown to exert deleterious effects on mitochondria from both liver[80] and insulinoma[74] such a mode of action of phosphoenolpyruvate seems unlikely.

A more straightforward approach to studying Ca^{2+} transport by organelles is the direct monitoring of the free Ca^{2+} concentration in the medium surrounding the isolated fractions using Ca^{2+} selective electrodes. This method requires relatively large amounts of tissue and is not easily practicable with organelles obtained from pancreatic islets. Therefore, we have applied this technique to subcellular fractions of a transplantable and glucose sensitive rat insulinoma[44,63].

Ca^{2+} sequestration by the mitochondria started immediately following their addition to the medium and resulted in a rapid decrease in ambient $[Ca^{2+}]$ (fig. 6). In the absence of Na^+ and Mg^{2+} an extramitochondrial Ca^{2+} steady state of around 0.3 μM was maintained. When a pulse addition of Ca^{2+} was made, the mitochondria rapidly restored the previous ambient $[Ca^{2+}]$. Several increments in medium Na^+ induced a stepwise increase in $[Ca^{2+}]$ until a plateau was reached at 10 mM Na^+. This higher Ca^{2+} steady state displayed the characteristics of a true mitochondrial 'set-point'[5,74]. Hence whenever alternate additions of Ca^{2+} or EGTA were made, the organelle restored $[Ca^{2+}]$ to the same level. As in other tissues, Na^+ was demonstrated to activate the Ca^{2+} efflux component of the mitochondrial Ca^{2+} cycle[16]. In addition, other ions, i.e. Mg^{2+} and H^+ were shown to influence the mitochondrial Ca^{2+} set-point[74]. Thus in a medium with a similar ionic composition to the cytosol of many cell types with respect to K^+ (110 mM), Na^+ (10 mM), Mg^{2+} (1 mM), at a pH of 7.0, rat insulinoma mitochondria buffered ambient $[Ca^{2+}]$ at around 1.0 μM[74] (fig. 7C). This Ca^{2+} level is one order of magnitude higher than the resting cytosolic Ca^{2+} (see fig. 4), and therefore the question arises as to the function of the mitochondria in Ca^{2+} homeostasis in the intact cell. Under similar experimental conditions rat insulinoma microsomes behave quite differently (fig. 7A). This fraction, whose Ca^{2+}-transporting activity correlated with a marker enzyme of the endoplasmic reticulum[76] lowered ambient $[Ca^{2+}]$ within 5–10 min to the range of $[Ca^{2+}]_i$ in the intact cell (0.1 μM). This Ca^{2+} steady state required the presence of Mg ATP and was modulated by ADP. Thus the addition of ADP (at concentrations present in cells) induced a dose-dependent Ca^{2+} efflux from the microsomes until a higher Ca^{2+} steady state was reached[76]. Furthermore, when medium ATP was markedly reduced (by the combined addition of glucose and hexokinase) the Ca^{2+} accumulated was released[76]. The findings suggest that variations in the cellular ATP/ADP ratio may modulate the Ca^{2+} transporting activity of the endoplasmic reticulum and change cytosolic $[Ca^{2+}]$ and consequently insulin release. It is interesting to note that this could be a means of coupling some metabolic and cationic events during nu-

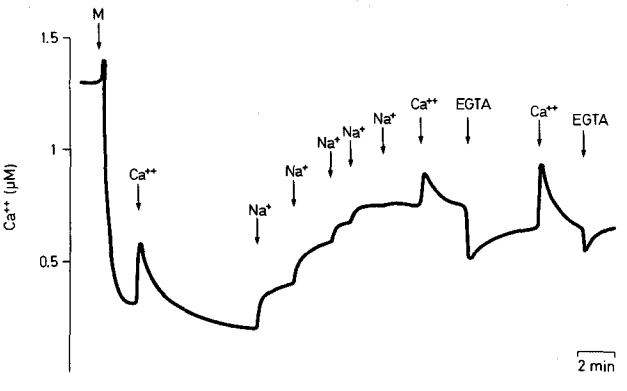

Figure 6. Maintenance of a steady state ambient free Ca^{2+} concentration by rat insulinoma mitochondria: effect of Na^+. Mitochondria were incubated at 30 °C, pH 7.0, in 200 μl of a buffer containing 110 mM KCl, 2 mM KH_2PO_4, 25 mM Hepes, 5 mM succinate, 0.2 mM Na_2ATP. Where indicated, 10 nmol/mg protein of $CaCl_2$ and 15 nmol/mg protein of EGTA were added. Na^+ indicates pulse additions of NaCl which increased the final NaCl concentration of the buffer by 2.5 mM per addition. M, represents the addition of mitochondria (final concentration, 0.3 mg protein/ml). Medium $[Ca^{2+}]$ was continuously recorded with a Ca^{2+}-selective electrode. For more details, see Prentki et al.[74].

trient-induced insulin release[76]. Indeed, it has been reported that 1 min after glucose addition to pancreatic islets the ATP/ADP ratio is diminished[61]. In contrast to ADP, neither Na^+, cAMP nor 3-isobutyl-1-methylxanthine altered Ca^{2+} fluxes by the microsomes[76].

Insulin-containing secretory granules, in contradistinction to the mitochondria or the endoplasmic reticulum, did not lower [Ca^{2+}] and were unable to take up Ca^{2+} (after pulse addition) (fig. 7D). However, the large granular calcium content[46,76] could be released by the Ca^{2+} ionophore A23187[76] (fig. 7D). It can be argued that secretory granules do not participate in the short-term regulation of cytosolic Ca^{2+}. This agrees with the conclusion from some studies[13,45] but not others[3,52] employing $^{45}Ca^{2+}$.

The Ca^{2+} electrode approach offers the opportunity of testing the coordinated function of isolated organelles. Accordingly, when mitochondria, microsomes and secretory granules were incubated together, the microsomes, but not the other organelles, buffered ambient [Ca^{2+}] again in the range of cytosolic [Ca^{2+}]. Nonetheless, mitochondria appear to be important in the rate of lowering of Ca^{2+} to the submicromolar level, whereas microsomes further reduce [Ca^{2+}] to the resting level (M. Prentki et al., submitted for publication).

The second messenger function of inositol 1,4,5-trisphosphate

Several neurotransmitters, growth factors and hormones have been shown to mobilize stored Ca^{2+}[7,11,18,69,85,95]. A characteristic feature of these Ca^{2+} agonists is that they all enhance the turnover of phosphoinositides[7,67]. Until recently the link between membrane receptor activation and the organelle(s) from which Ca^{2+} is released was unknown.

The finding that some of these agonists induce a rapid and Ca^{2+} independent breakdown of the polyphosphoinositides leading to the accumulation of the water soluble compounds, inositol 1,4-bisphosphate (IP_2) and inositol 1,4,5-trisphosphate (IP_3) has provided a hint to the mechanism ivolved[6,78,84,95]. Subsequently it was demonstrated in permeabilized pancreatic acinar cells[92] that the addition of IP_3 released Ca^{2+} from a non-mitochondrial pool. These findings have been confirmed in liver[15,49] and in RINm5F cells[9].The converging evidence from these and other studies strongly suggests that IP_3 is the second messenger of the Ca^{2+} mobilizing agonists[7,15,49,75,77,92].

The identification of the target organelle for IP_3 has been achieved in rat insulinoma[75,77]. When IP_3 was added to the microsomal fraction (enriched in endoplasmic reticulum) a rapid Ca^{2+} release was observed (fig. 7A). At a maximal concentration of IP_3, medium [Ca^{2+}] rose from 0.1 μM to about 0.2 μM followed by Ca^{2+} re-uptake. These values compare favorably to basal and stimulated [Ca^{2+}]$_i$ in insulin secreting cells[81,102]. The effect was half-maximal and maximal at 3 μM and 10 μM IP_3, respectively[77]. The action is specific for the trisphosphate derivative since myo-inositol and inositol monophosphate (IP_1) (fig. 7A) and IP_2[77] were ineffective.

An interesting feature of IP_3 action was a desensitization phenomenon apparent when a second IP_3 pulse was introduced (fig. 7A). Although the exact nature of the desensitization is unknown, it may be related to the slow degradation of IP_3 by the microsomes[77]. Hence, the resensitization to the Ca^{2+} mobilizing molecule may depend on the completion of its degradation.

It was also found that IP_3 did not release Ca^{2+} from microsomes incubated in the absence of ATP and exposed to the Ca^{2+} ionophore A23187, conditions which deplete the vesicles of Ca^{2+} (fig. 7B). The insignificant and immediate elevation in [Ca^{2+}] following IP_3 addition (fig. 7B) was due to a small Ca^{2+} contamination of the compound amounting to less than 10% of the effect shown in figure 7A. Similarly, IP_3 did not release Ca^{2+} from the mitochondria (fig. 7C) or the secretory granules (fig. 7D) under the experimental conditions where it was effective on the microsomes (fig. 7A). Two main arguments suggest that IP_3 releases Ca^{2+} from the endoplasmic reticulum and not from plasma membrane vesicles contaminating the microsomes. First, the Ca^{2+} transporting activity of the microsomes correlated with a marker enzyme of the endoplasmic reticulum but not of the plasma membrane[76,77]. Second, IP_3 has been documented to mobilize Ca^{2+} from permeabilized cells[15,49,92], including the insulinoma cell line RINm5F[9]. In these preparations the plasma membrane cannot

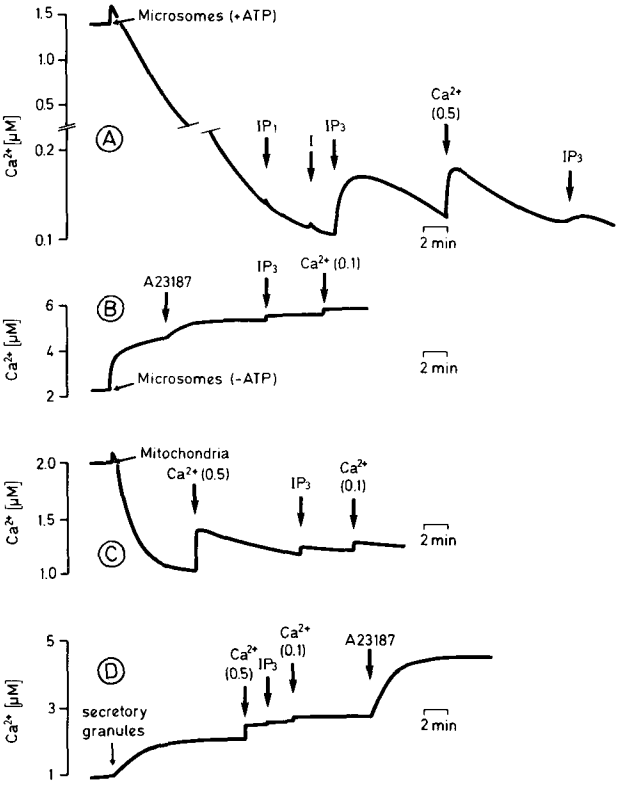

Figure 7. Ca^{2+} transport by rat insulinoma organelles and the effect of inositol 1,4,5-trisphosphate (IP_3). Organelles were incubated as described in the legend to figure 6, in a medium containing 1 mM $MgCl_2$, 1 mM Mg ATP, 2 mM creatine phosphate and 50 μg/ml of creatine kinase. Where indicated, microsomes (0.5 mg protein/ml), mitochrondria 0.25 mg protein/ml), and secretory granules (0.125 mg protein/ml) were added. Ca^{2+}, denotes the addition of the ion at the amount indicated in parentheses (nmol). A23187 (1 μg/ml), IP_3 (10 μM), inositolmonophosphate (IP_1) (10 μM) and myoinositol (10 μM) were added as indicated. For details see Prentki et al.[77].

function as a vesicular Ca^{2+} store. It can, therefore be concluded that IP_3 mobilizes Ca^{2+} from a vesicular and Mg ATP dependent pool, which is most probably the endoplasmic reticulum.

The evidence for a second messenger function of IP_3 in insulin release will now be discussed.

Acetylcholine or carbamylcholine stimulates insulin release from islets[58,96,99] and RINm5F cells[75]. In the latter cell type the activation of the muscarinic receptors by carbamylcholine raises $[Ca^{2+}]_i$, even in the absence of extracellular Ca^{2+}, which indicates that at least part of this $[Ca^{2+}]$ rise is due to Ca^{2+} mobilization[75]. Both carbamylcholine and glucose have been reported to enhance the generation of inositol phosphate (IP_1 plus IP_2 plus IP_3)[8]. The breakdown of phosphphatidylinositol 4, 5-bisphosphate, has only been studied in detail in glucose-stimulated islets[27,54], but it appears that the actions of glucose on phospholipid turnover are more dependent on extracellular Ca^{2+}[8,27,54] than those of carbamylcholine[8]. These findings taken together, with the observation that IP_3 releases stored Ca^{2+}, strongly favor the hypothesis that IP_3 is one of the intracellular mediators of certain secretagogues[9]. The reported stimulation of de novo synthesis of polyphosphoinositides by glucose may be important for sustained stimulation of insulin release[28].

The suggested role of IP_3 in Ca^{2+} homeostasis in the B-cell is illustrated in figure 8. Hence, activation of phospholipase C by agonist-receptor interaction leads to phosphatidylinositol 4, 5-bisphosphate breakdown and the production of both diacylglycerol and IP_3. In turn, IP_3, which is a hydrophilic molecule, reaches the endoplasmic reticulum and by an as yet undefined mechanism, elicits the release of Ca^{2+}. IP_3 probably stimulates an independent Ca^{2+} efflux component of this pool (M. Prentki et al., manuscript submitted for publication). The resulting rise in $[Ca^{2+}]_i$ triggers insulin release by exoytosis. Little is known concerning the mode by which Ca^{2+} elicits exocytosis although calmodulin-independent[14] and Ca^{2+}-calmodulin-mediated protein phosphorylation[21,36,86,87] has been implicated (for review, Sharp et al.[89]). The other product of phosphoinositide breakdown, diacylglycerol[7,26] through its action on protein kinase C[57,68,94] may also participate in the activation of the release machinery to elicit the optimal cellular response[29,62,105].

Although not included in figure 8, Ca^{2+} permeability changes at the plasma membrane also contribute to raise $[Ca^{2+}]_i$. As already discussed, both voltage-dependent and receptor-activated Ca^{2+} channels may be opened during secretagogue stimulation. The elevated cytosolic $[Ca^{2+}]$ could raise the mitochondrial matrix $[Ca^{2+}]$ and thereby activate mitochondirial Ca^{2+} sensitive enzymes, (e.g. pyruvate dehydrogenase) leading to a stimulation of oxidative metabolism in order to sustain the secretory process. The recovery to the non-stimulated state is thought to be mediated by Ca^{2+} transporting devices in the endoplasmic reticulum (a Ca^{2+}-ATPase) and in the plasma membrane (a Ca^{2+}-ATPase and a Na^+/Ca^{2+} countertransport).

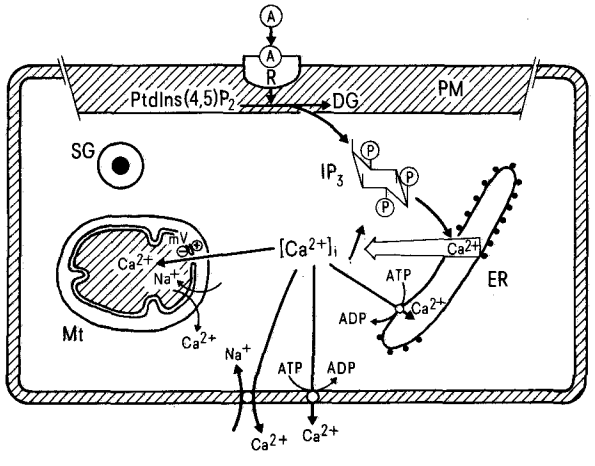

Figure 8. Inositol 1, 4, 5 trisphosphate and the regulation of cytosolic Ca^{2+} homeostasis in the B-cell. The abbreviations are: A, agonist, e.g. carbamylcholine; R, membrane receptor; Ptd Ins (4,5)P_2, phosphatidylinositol, 4, 5-bisphosphate; DG, diacylglycerol; PM, plasma membrane; IP_3, inositol 1, 4, 5-trisphosphate; ER, endoplasmic reticulum; Mt, mitochondria; SG, secretory granules.

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