Dissociation between exocytosis and Ca^{2+}-channel activity in mouse pancreatic β-cells stimulated with calmidazolium (compound R24571)

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Received 29 June 1995

Abstract Calmidazolium, a calmodulin inhibitor, suppressed influx of Ca^{2+} through voltage-gated Ca^{2+} channels in mouse pancreatic β-cells. Despite this fact, calmidazolium stimulated insulin release from β-cells at basal glucose concentration. This effect was not mediated by protein kinase C (PKC), since it persisted in PKC-depleted cells. R_pcAMPS significantly attenuated the calmidazolium-stimulated insulin secretion, indicating that calmidazolium acts, at least partly, through PKA. The compound also stimulated insulin secretion from electropermeabilized β-cells, indicating effects on distal steps in the stimulus–secretion coupling. The use of calmidazolium offers possibilities to investigate the mechanisms activating exocytosis under conditions where the cytoplasmic-free Ca^{2+} concentration does not increase.

Key words: Voltage-gated Ca^{2+} channel; Calmidazolium; Insulin secretion; Protein kinase A

1. Introduction

In the pancreatic β-cell, the physiological secretagogue glucose causes insulin release through a complex mechanism involving closure of ATP-regulated K^+ channels, depolarization, opening of voltage-gated L-type Ca^{2+} channels and, thus, increase in $[Ca^{2+}]_i$ [1,2]. A tight coupling is believed to exist between exocytosis of insulin-containing secretory granules and an increase in the cytoplasmic-free Ca^{2+} concentration, $[Ca^{2+}]_i$. Substances that block influx of Ca^{2+} into the β-cell, e.g. blockers of voltage-gated L-type Ca^{2+} channels, can usually be relied upon to inhibit hormone secretion [3]. Some compounds, e.g., phorbol esters, however, are able to promote vigorous insulin secretion from β-cells despite only minor increments in the Ca^{2+} current [4].

Calmidazolium, which suppressed Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels in clonal, insulin-producing RINm5F-cells, concomitantly inhibited insulin release from those cells [5]. However, preliminary data surprisingly indicated that calmidazolium actually stimulated insulin secretion from pancreatic β-cells isolated from the ob/ob mouse [6]. Calmidazolium (compound R24571) has been shown to be an inhibitor of the calmodulin-dependent Ca^{2+}-transport ATPase in red blood cells [7]. The compound also inhibits activation of brain phosphodiesterase by calmodulin, with a $500 \times$ norm higher potency than trifluoroperazine [8]. The aim of the present study was to clarify whether calmidazolium can be used to promote exocytosis under conditions where $[Ca^{2+}]_i$ remains unchanged, and, if so, the underlying molecular mechanisms.

2. Materials and methods

2.1. Chemicals, statistics

All reagents were of analytical grade and redistilled deionized water was used. Bovine serum albumin fraction V, Fura-2/acetoxymethylester and calmidazolium were from Sigma. Student's *t*-test for unpaired data was used throughout the study for determination of statistically significant differences between groups.

2.2. Media

The medium used for measurements of $[Ca^{2+}]_i$ was a HEPES buffer (pH 7.4) containing 1.28 mM Ca^{2+}, unless otherwise stated, and with Cl^- as the sole anion [9]. Bovine serum albumin, at a concentration of 1 mg/ml, was added to the medium. For the studies of permeabilized cells a HEPES buffer, pH 7.0, was used; this contained (in mM) 110 KCl, 10 NaCl, 2 KH_2PO_4, 1 $MgCl_2$ and 0.5 mg/ml of bovine serum albumin. This buffer was supplemented with 2 mM ATP and an ATP-regenerating system, consisting of 10 mM phosphocreatine and 20 U creatine kinase/ml. The buffer was also supplemented with 5 mM EGTA and Ca^{2+} in appropriate amounts to give the various desired Ca^{2+} concentrations. For the measurements of glucose oxidation and utilization and for the measurements of insulin secretion during static incubation, a Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 3 3.3 mM glucose, was used.

2.3. Animals and preparation of islet cells

Adult obese hyperglycemic mice (gene symbol ob/ob) of both sexes, were obtained from a local non-inbred colony and starved overnight. The animals were killed by decapitation and the islets isolated by a collagenase technique [10]. The islets of these mice contain >90% β-cells [11]. A cell suspension was prepared and washed essentially as previously described [12]. The cells were resuspended in RPMI 1640 culture medium (Flow Laboratories, UK), containing 11 mM glucose supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 60 μg/ml gentamicin. The cell suspension was then either plated on coverslips or maintained in suspension by constant agitation. Islets from male Wistar rats were also isolated by a collagenase technique and were cultured overnight in RPMI 1640 culture medium.

2.4. Measurements of $[Ca^{2+}]_i$

Cells attached to coverslips were incubated with 1.5 μM of the intracellular fluorescent Ca^{2+} indicator fura-2, in its permeable acetoxymethylester form, for 15 min at 37°C. During incubation, cells were also exposed to either 5 μM calmidazolium or 0.1% of the calmidazolium solvent Me_2SO. In addition, calmidazolium or Me_2SO was present during subsequent experiments. $[Ca^{2+}]_i$ was measured in small aggregates of mouse pancreatic β-cells plated on coverslips. These measurements were carried out essentially as previously reported [13], using a SPEX fluorolog-2 CM1T111 system connected to an inverted microscope (Zeiss, Axiovert 35M). Conversion of fluorescence values to $[Ca^{2+}]_i$ was made according to a previously described equation [14].

2.5. Patch-clamp experiments

Subsequent to culture for 24 h, cells were washed with a solution composed as follows (mM): choline-Cl (138), KCl (5.6), $MgCl_2$ (1.2), $CaCl_2$ [10], tetraethylammonium-chloride [10], HEPES [5] at pH 7.4. Cells were covered with 350 μl of the described solution and treated

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Abbreviations: $[Ca^{2+}]_i$, cytoplasmic-free Ca^{2+} concentration; Me_2SO, dimethylsulphoxide; NMDG, *N*-methyl-D-glucamine; PKA, protein kinase A; PKC, protein kinase C; TPA, phorbol 12-myristate 13-acetate.

SSDI 0014-5793(95)00774-1

with 5 μM calmidazolium for at least 15 min at 37°C. The same amount of Me_2SO was added to the control dishes. The pipette solution contained (mM): *N*-methyl-D-glucamine (NMDG) (150), HCl (110), $MgCl_2$ [1], $CaCl_2$ [2], EGTA [10], Mg-ATP [3], HEPES [5] at pH 7.15. All experiments were performed at room temperature (22–24°C). NMDG was substituted for K^+ in the pipette solution, in order to block outward directed K^+ currents. Likewise, choline-Cl was substituted for NaCl in the extracellular medium, in order to block inward directed Na^+ currents. The whole-cell configuration of the patch-clamp technique [15] was used, utilizing an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA). Voltage-steps were generated, digitized and stored in a computer (IBM AT-clone), using the program pClamp (Axon Instruments) and Labmaster ADC (Scientific Solutions, Solon, OH). The current responses were filtered at 1 kHz, Bessel filter (−3 dB point). The pulse protocol is given in the figure legend.

2.6. Measurements of glucose oxidation and glucose utilization

Islets from ob/ob mice were incubated in Krebs-Ringer-bicarbonate buffer containing radioactively labelled glucose ([5-^{3}H]glucose and [U-^{14}C]glucose) purchased from New England Nuclear (Boston, MA). In order to determine glucose oxidation and utilization in the same individual experiment, we incubated the islets in the presence of both ^{3}H- and ^{14}C-labelled glucose. The islets were incubated in 100 μl of medium containing the labelled glucose and for each group of islets a second vial containing 100 μl of identical medium was incubated without islets and used to estimate background yield of ^3H_2O and $^{14}CO_2$. Half of the islets were exposed to 5 μM calmidazolium and half to a corresponding amount of Me_2SO as control. Each vial was placed inside a scintillation bottle and the bottle was gassed with 95% O_2:5% CO_2 and then sealed. The incubations were done in triplicate, each with 20 islets. After incubation for 60 min at 37°C, 100 μl of 10% perchloric acid was injected into the vials, in order to stop cellular metabolism. 500 μl Hyamine and 500 μl H_2O were injected into the scintillation bottles to absorb $^{14}CO_2$ and ^3H_2O. After overnight incubation, the incubation vials were removed from the scintillation bottles and 5 ml scintillation fluid was added into the bottle, which was then counted for radioactivity in a $^3H/^{14}C$ channel. The utilization and oxidation of glucose were measured from the yield of ^3H_2O and $^{14}CO_2$, respectively.

2.7. Measurements of insulin release

Kinetics of insulin release were evaluated by perifusing dispersed β-cells or intact mouse islets mixed with Bio-Gel P4 polyacrylamide beads (Bio-Rad) in a 0.5-ml column at 37°C [16,17]. The flow rate was ~250 μl/min and 2-min fractions were collected and analysed for insulin radioimmunologically, using crystalline rat insulin as a reference. Insulin release from permeabilized cells was studied essentially as in Nilsson et al. [11]. Briefly, cells were added to solutions of specified Ca^{2+} concentrations containing the test substance. The Ca^{2+} concentrations were checked before the experiments with a Ca^{2+}-sensitive mini-electrode. Permeabilized cells were incubated during gentle agitation for 30 min at 37°C. After incubation, cell suspensions were centrifuged and samples were taken from the supernatant and assayed for insulin.

Insulin release from intact islets from male Wistar rats was studied using static incubation of groups of 3 islets for 1 h in the presence or absence of calmidazolium.

3. Results

3.1. Measurements of $[Ca^{2+}]_i$

When mouse β-cells were stimulated with 25 mM KCl, in the presence of Me_2SO (0.1%) only, there was a large increase in $[Ca^{2+}]_i$ but, when the experiments were performed in the presence of 5 μM calmidazolium, the increase in $[Ca^{2+}]_i$ was significantly attenuated (Fig. 1A).

3.2. Patch-clamp experiments

To investigate if the decrease in depolarization-induced elevation in $[Ca^{2+}]_i$ was due to direct inhibition of the Ca^{2+} current through voltage-gated L-type Ca^{2+} channels, patch-clamp recordings were performed. As exemplified in Fig. 1B, pretreat-

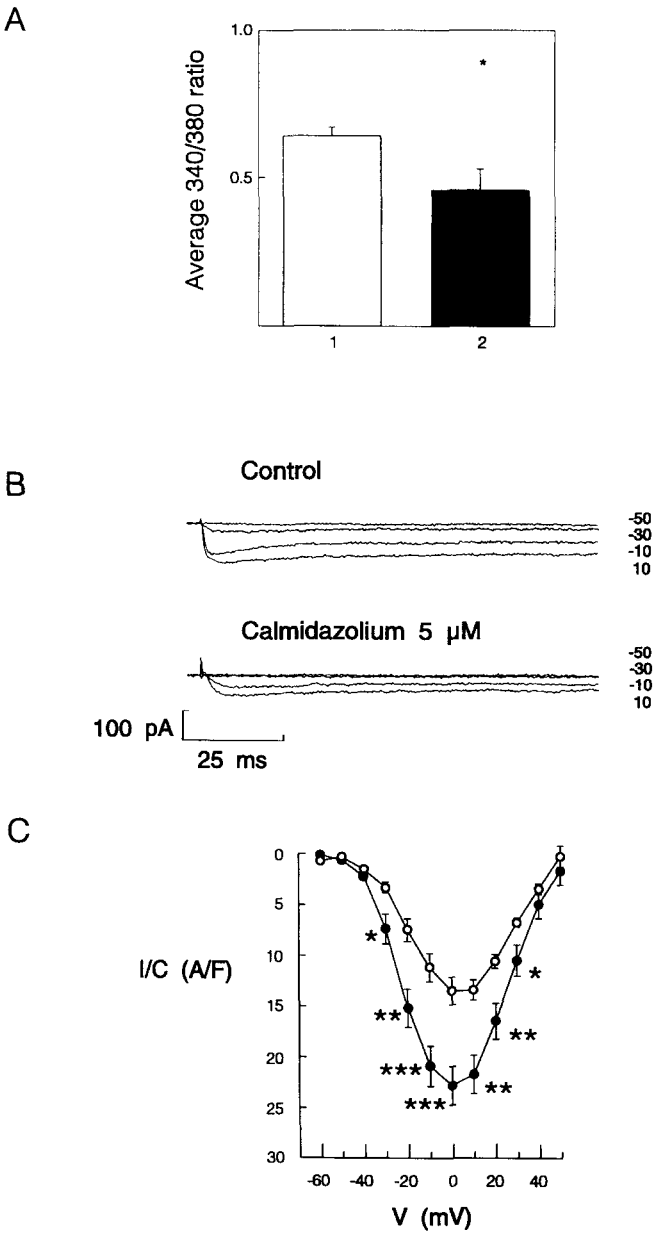

Fig. 1. Effect of calmidazolium on depolarization-induced increases in $[Ca^{2+}]_i$ measured with fura-2 and on Ca^{2+} currents, registered with the patch-clamp technique. (A) Mouse β-cell aggregates were stimulated with 25 mM KCl in the presence of 3 mM glucose and $[Ca^{2+}]_i$ was monitored. Results are expressed as average 340/380-ratio for the first 180 s after stimulation. This average value was obtained by using the analysis feature of the SPEX Cation Measurement program to calculate the 'area under the curve' for these 180 s and dividing by 180. Mean values ± S.E.M. $^*P < 0.05$. (1) Control experiments performed in the presence of 0.1% Me_2SO (7 experiments performed on 4 different cell preparations). (2) Experiments performed in the presence of 5 μM calmidazolium (7 experiments performed on three different cell preparations). (B) The whole-cell configuration of the patch-clamp technique was established ~1 min before starting the pulse protocol. Current traces from control (Me_2SO) and calmidazolium-treated β-cells. The depolarizing steps were evoked to the membrane potentials indicated in the figure, from a holding potential of −70 mV. (C) Compiled data of the I–V relationship. Open circles represent calmidazolium-treated cells and filled circles represent control cells. To compensate for variation in cell size, currents are expressed as current densities (I/C), which were obtained by normalizing the current amplitude (I) to cell capacitance (C). Mean values ± S.E.M., $n = 16$ for calmidazolium-treated cells and $n = 22$ for control cells. Results from at least three different preparations of β-cells. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

ment of β-cells (for 15 min) with 5 μM of calmidazolium resulted in a clear-cut decrease in whole-cell Ca^{2+} currents (only falling part of *I–V* relationship shown). The effect of calmidazolium was most pronounced at voltages around 0 mV (Fig. 1C).

3.3. Measurements of insulin release

Since normal β-cells displayed a decreased influx of Ca^{2+} through voltage-gated Ca^{2+} channels in the presence of calmidazolium, it is logical to assume that this effect should be parallelled by inhibition of insulin release, as indeed was found to be the case in clonal insulin-producing RINm5F-cells [5]. However, β-cells treated with calmidazolium had an average basal insulin secretion that was 21 × higher than that from control β-cells (data not shown). In this context, it should be kept in mind, that basal $[Ca^{2+}]_i$ in the calmidazolium-treated cells was not significantly different from that in control cells (42 ± 9 vs. 49 ± 11 nM, $n = 3$ and 4, respectively). Since the β-cell suspension was divided into two equal portions just prior to the perifusion experiment, the cell number in the two groups should be similar and can not explain the great difference in basal insulin secretion. Experiments were also performed to investigate if calmidazolium activates insulin secretion from β-cells in the more physiological framework of the intact pancreatic islet. It was demonstrated that calmidazolium significantly stimulated insulin release (250 ± 55% above average basal secretion, $n = 5$, $P < 0.01$) at basal glucose in intact pancreatic islets from ob/ob-mouse. Also, in experiments on intact islets from male Wistar rats, 5 μM calmidazolium significantly stimulated insulin secretion at basal glucose (38.5 ± 1.8 μU/islet/h [calmidazolium group] compared with 14.5 μU/islet/h [control group], $n = 3$, $P < 0.05$).

Since PKC activation with phorbol ester causes a substantial stimulation of insulin secretion even at basal glucose levels [18], it was investigated whether calmidazolium stimulation of insulin release is mediated by PKC. Suspensions of β-cells from ob/ob-mouse were treated for 24 h with 200 nM TPA, in order to downregulate protein kinase C (PKC) activity [18], or with 0.1% Me_2SO as control. The cell suspensions were then stimulated by 5 μM calmidazolium for 10 min. There was no difference in insulin secretion in the PKC-downregulated group, compared with the control group (data not shown), speaking against PKC as the mediator of the calmidazolium effect. Phorbol ester-stimulated insulin release from β-cells is markedly potentiated by prior glucose stimulation [18]. Experiments were performed to study if the calmidazolium-induced insulin secretion could be potentiated by initial glucose stimulation. It was found that the relative insulin secretion caused by calmidazolium was unaffected by prior exposure of the β-cells to glucose (Fig. 2A,B), again suggesting that calmidazolium is stimulating hormone release through other mechanisms than those activated by phorbol ester. The calmidazolium effect was partially reversible during the 18 min of observation after cessation of calmidazolium stimulation (Fig. 2A,B).

Since calmidazolium potently stimulated insulin release from intact β-cells, without increasing $[Ca^{2+}]_i$, it was of interest to study if the compound also caused insulin secretion from permeabilized β-cells. In such cells, the plasma membrane electrical potential is short-circuited and rapid equilibration of ions and small molecules occurs. Cellular organelles and microtubular-microfilamentous structures, however, are left intact. As is

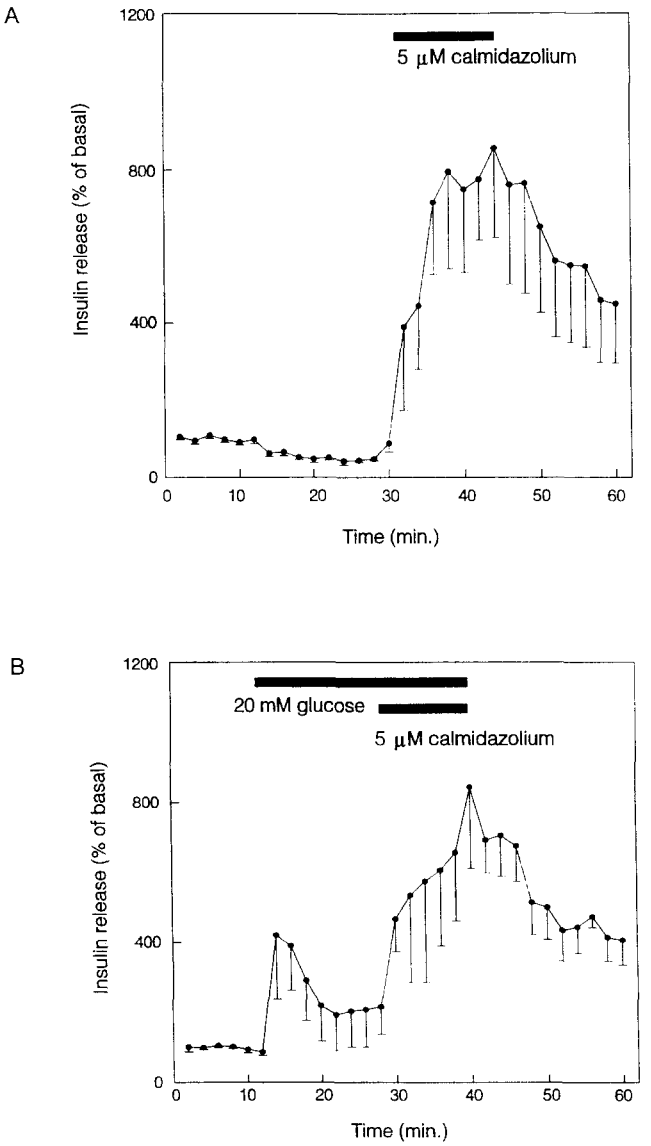

Fig. 2. Effect of glucose on subsequent calmidazolium stimulation of insulin release from mouse β-cell suspensions. This figure also shows the effect of calmidazolium withdrawal. (A) 5 μM calmidazolium is added in the presence of 3 mM glucose. (B) β-cells are first stimulated with 20 mM glucose and then with 5 μM calmidazolium. Average insulin release during the first 10 min was taken as 100% and all values are given relative to this. Mean values ± S.E.M. ($n = 3$–4).

shown in Fig. 3, 5 μM calmidazolium significantly increased insulin release (B) compared with control experiments (A) in permeabilized β-cells incubated in buffers where the Ca^{2+} concentration was clamped to 100 nM. Cells incubated in a buffer containing 10 nM TPA and 100 μM Ca^{2+}, which potently stimulates insulin secretion, were used as a positive control of the experimental system (C).

In order to evaluate if calmidazolium was stimulating insulin release by interacting with the PKA-signalling system, β-cells were treated with R_pcAMPS, an inhibitor of PKA [19]. As is demonstrated in Fig. 4, after 20 min of calmidazolium stimulation, it was found that in β-cells treated with R_pcAMPS, insulin release was stimulated by 865 ± 174% (A) compared with 2274 ± 561% (B) in the control group. The difference is statisti-

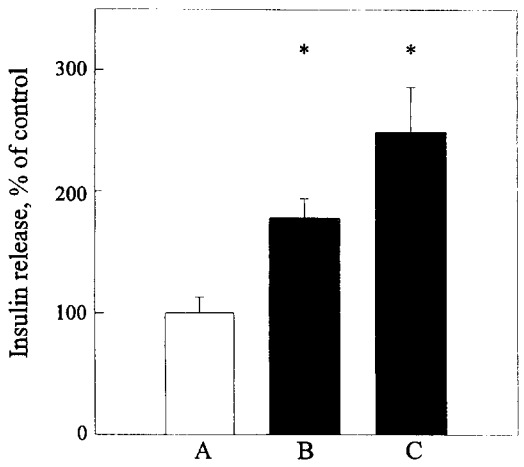

Fig. 3. Effects of calmidazolium and TPA+high Ca^{2+} on insulin release from permeabilized mouse β-cells. Results are indicated by vertical bars. (A) Control cells incubated in buffer containing 0.1% Me_2SO and 100 nM Ca^{2+}. (B) Cells incubated in a buffer containing 5 μM calmidazolium and 100 nM Ca^{2+}. (C) Cells incubated in a buffer containing 10 nM TPA and 100 μM Ca^{2+}, as a positive control of the experimental system. Average insulin release in the Me_2SO control group in each experiment was taken as 100% and insulin release in all groups was then normalized with respect to this average secretion. Mean values ± S.E.M. Statistical significances were calculated using Student's t test for unpaired data, applying the Bonferroni correction for multiple comparisons. *$P < 0.01$ ($n = 6$, three different cell preparations). When evaluating statistical significances, groups B and C were compared with the control group A.

cally significant ($P < 0.05$) and suggests that the stimulatory effect of calmidazolium is at least in part mediated by the cAMP-PKA system.

3.4. Measurements of glucose oxidation and glucose utilization

In order to exclude unspecific toxic actions of calmidazolium on the β-cell, the effect of the compound on glucose oxidation and utilization was measured. Glucose oxidation was 12.6 ± 1.9 pmol/islet/h in islets exposed to 5 μM calmidazolium compared with 10.9 ± 1.4 pmol/islet/h in control islets. Glucose utilization was 83.3 ± 8.0 pmol/islet/h (calmidazolium group) and 73.5 ± 5.7 pmol/islet/h (control group). The differences between calmidazolium-treated and control islets regarding the two aspects of glucose metabolism were not statistically significant. Exclusion of Trypan blue was the same in calmidazolium-treated β-cells as in control cells (data not shown).

4. Discussion

We demonstrate that influx of Ca^{2+} through voltage-gated L-type Ca^{2+} channels in mouse pancreatic β-cells is attenuated by calmidazolium. This was evident both from direct measurements of voltage-gated Ca^{2+}-channel activity and changes in $[Ca^{2+}]_i$ in response to K^+ depolarization. The results are in accordance with previously published data by Safayihi et al. [20], Li et al. [21] and Kindmark et al. [5], which showed that the calmodulin antagonists CGS9343B [20,21] and calmidazolium [5] inhibit KCl-induced increases in $[Ca^{2+}]_i$ in insulin-producing cell lines. Calmidazolium is likely to inhibit not only Ca^{2+} influx but also outward transport of Ca^{2+} by the plasma membrane Ca^{2+} pump, a calmodulin-dependent enzyme [22]. This fact could explain why the inhibition of Ca^{2+} influx through voltage-gated Ca^{2+} channels was more pronounced (Fig. 1C) than the attenuation of depolarization-induced rise in $[Ca^{2+}]_i$ (Fig. 1A). The latter effect is the net result of both influx and extrusion of Ca^{2+} ions. The mechanism by which calmidazolium inhibits Ca^{2+} flux through voltage-gated Ca^{2+} channels is not clear. It has, however, been demonstrated that calmidazolium, at the concentrations used in the present study, displaces the Ca^{2+}-channel antagonists nitrendipine and diltiazem from their binding sites in rat cerebral cortex homogenate [23]. Thus, calmidazolium could inhibit Ca^{2+} influx by direct interaction with the channel protein. Furthermore, the voltage-gated Ca^{2+} channel is a substrate for phosphorylation [24] and interference with Ca^{2+}-calmodulin-dependent channel protein phosphorylation cannot be ruled out as a mechanism for calmidazolium-induced inhibition of Ca^{2+} influx. Indeed, in a recent study, Ca^{2+} currents in smooth muscle cells were enhanced by calmodulin-dependent protein kinase II in a Ca^{2+}-dependent manner [25].

Since influx of Ca^{2+} into normal mouse β-cells was reduced in the presence of calmidazolium, it was of interest to investigate the effects of the compound on insulin release. Unexpectedly, basal insulin secretion was greatly increased in the presence of the compound. It has been demonstrated that 10 μM calmidazolium potently stimulates secretion of ATP from human platelets [26]. In that study, however, the calmodulin antagonist also increased $[Ca^{2+}]_i$ and it was suggested that calmidazolium could possess ionophoretic properties. With regard to normal β-cells, the stimulatory effect of calmidazolium on insulin release is unlikely to be caused by unspecific permeabilization of the plasma membrane and consequent leakage of hormone to the extracellular space. After 15 min of incubation in the presence of calmidazolium, basal $[Ca^{2+}]_i$ remained normal and exclusion of Trypan blue was the same as in control cells. Also speaking against a general toxic effect of calmidazolium on insulin-producing cells, is the fact that 10 μM of the compound did not affect glucose oxidation or utilization in the present study, or in RINm5F-cells in a previous study [5]. The fact that islets from the ob/ob mouse respond with insulin secretion during stimulation with a substance, which is a calmodulin antagonist and which blocks voltage-gated Ca^{2+} channels, is in agreement with previously published data, showing that both the calmodulin antagonist trifluoroperazine and Ca^{2+}-channel antagonists verapamil and nifedipine stimulate insulin release at basal glucose in islets from this animal model [27]. However, since calmidazolium also stimulated insulin secretion in rat islets, this response may be a more general feature of normal, differentiated β-cells. RINm5F-cells are clonal tumor cells that exhibit a different stimulus–secretion coupling than normal β-cells, which may account for the fact that calmidazolium has no effect on basal insulin release in this cell line [5].

Agonists stimulating PKC can induce insulin secretion even at basal glucose concentrations [18] and experiments were therefore performed to determine if calmidazolium was acting through this enzyme. Insulin release in the presence of calmidazolium was the same in β-cells, whether subjected to PKC downregulation or not. Downregulation leaves a residual PKC activity, corresponding to ~16% of normal [18], which may be part of the calmidazolium-signalling pathway. However, further support for the fact that PKC, at least those isoforms that

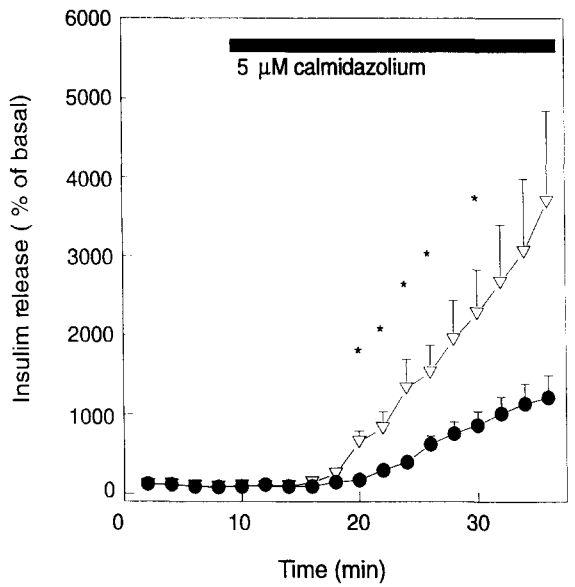

Fig. 4. Effect of R_pcAMPS treatment of mouse β-cell suspensions on subsequent calmidazolium stimulation of insulin release. R_pcAMPS present for ~15 min before addition of calmidazolium and then throughout the experiment (filled circles). Control group in which a corresponding volume of the R_pcAMPS-solvent water was added (open taken as 100% and all values are given relative to this. Mean values ± S.E.M. ($n = 5$). $^*P < 0.05$. Experiments performed in the presence of 3 mM glucose.

are sensitive to phorbol ester, is not involved in the mechanism whereby calmidazolium stimulates insulin release is given by the results showing that this effect is not potentiated by prior glucose stimulation, which is true for TPA-induced insulin release [18].

Calmidazolium-stimulated insulin release is partially inhibitable by R_pcAMPS, suggesting that at least some of the insulinotropic effect of calmidazolium is mediated by the cAMP-PKA system. The compound may activate exocytosis either through direct interaction with PKA, or by raising the intracellular cAMP concentration. Calmidazolium has not been shown to interact directly with PKA, but as a calmodulin antagonist, calmidazolium may interfere with several steps of intracellular cAMP metabolism. In this context it is of interest to note that calmodulin has been implicated both in the regulation of cAMP formation, at the level of adenylate cyclase [28] and cAMP breakdown, at the level of cyclic nucleotide phosphodiesterase [29]. Hence, it is difficult to forecast the net effect of calmodulin inhibition on cAMP levels in the cell. Henquin has demonstrated that the calmodulin inhibitor trifluoroperazine slightly increases islet cell cAMP concentration in the absence of glucose [30]. A high concentration of the adenylate cyclase activator forskolin (20 μM), which increases intracellular cAMP concentrations 60-fold in islet cells, barely doubles insulin release at basal glucose [31]. However, in islets from ob/ob mice, cAMP-raising stimuli elicit a much more pronounced insulin release at basal glucose than in islets from lean mice [32].

It can again be concluded that an elevated $[Ca^{2+}]_i$ is not a prerequisite for activation of insulin release, a fact previously demonstrated for stimulators of PKC [33] and PKA [34]. Calmidazolium activates insulin secretion at resting $[Ca^{2+}]_i$ and although PKA seems to mediate part of the stimulatory effect, the complete understanding of calmidazolium-induced exocytosis of insulin in normal β-cells merits further investigations. A deeper understanding of the mechanisms involved should shed light on the molecular machinery directly regulating exocytosis in the pancreatic β-cell.

Acknowledgements: We thank Yvonne Strömberg and Tsegga Zeccarias for expert technical assistance with the radioimmunological measurements of insulin. Financial support was obtained from the Swedish Medical Research Council (03X-09890, 19x-00034, 04X-09891), the Juvenile Diabetes Foundation International, the Swedish Diabetes Association, the Nordic Insulin Foundation, Magnus Bergvalls Foundation, Lars Hiertas Memorial Foundation, NOVO Industry, Funds of the Karolinska Institute, Clas Groschinskys Memorial Foundation, the Swedish Society of Medicine and Förenade Liv Mutual Group Life Insurance Company.

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