Comparison of the effects of perchlorate and Bay K 8644 on the dynamics of cytoplasmic Ca^{2+} concentration and insulin secretion in mouse β -cells

Gerd LARSSON-NYRÉN* and Janove SEHLIN

Department of Histology and Cell Biology, Umeå University, S-901 87 Umeå, Sweden

Non-inbred *ob/ob* mice were used to study the dynamics of cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in isolated pancreatic β -cells using microfluorimetry with fura 2/AM as probe, and the dynamics of insulin secretion in isolated pancreatic islets. D-Glucose (20 mM) caused a transient peak increase in $[\text{Ca}^{2+}]_i$ which changed to either an oscillating or a flat, elevated phase. The lag-time before the first peak increase in $[\text{Ca}^{2+}]_i$ was markedly shortened by 12 mM ClO_4^- and the glucose-stimulated level of $[\text{Ca}^{2+}]_i$ after the first peak was clearly elevated by the anion. ClO_4^- also amplified K^+ -stimulated (20 mM) $[\text{Ca}^{2+}]_i$. ClO_4^- did not change the basal $[\text{Ca}^{2+}]_i$ at 3 mM glucose. Extracellular Ca^{2+} deficiency abolished the effect of high glucose and ClO_4^- on

$[\text{Ca}^{2+}]_i$. This suggests that ClO_4^- acts as an amplifier of transmembrane Ca^{2+} inflow. The L-type Ca^{2+} channel agonist, Bay K 8644 (0.01–1.0 μM), strictly reproduced all the effects of perchlorate on the glucose-stimulated β -cell $[\text{Ca}^{2+}]_i$. Both phases of insulin release (20 mM glucose) were markedly enhanced by ClO_4^- (12 mM) or Bay K 8644 (1.0 μM). The lag-time for glucose-stimulated insulin release was shortened by both agents. Taken together, these data strengthen the idea that perchlorate amplifies the glucose-stimulation of $[\text{Ca}^{2+}]_i$ and insulin release by directly modifying the function of the L-type Ca^{2+} channel. This effect can induce both a more prompt onset of and an amplified level of β -cell secretory activity.

INTRODUCTION

Stimulated insulin secretion shows anion selectivity. Chaotropic anions, such as perchlorate, thiocyanate and iodide, augment glucose-induced insulin secretion and labelled calcium ion uptake when added at low concentrations and are more effective than chloride in supporting β -cell activity, whereas other anions, like isethionate or acetate, do not augment β -cell activity and cannot replace chloride [1,2]. Among these anions, perchlorate exhibits the strongest potentiating effect on stimulated insulin release. The secretory effect is not exclusive to β -cells, as K^+ -induced secretion of growth hormone from isolated somatotrophic cells of rat pituitary was amplified by perchlorate [3]. It is known from a long series of studies on skeletal muscle, that perchlorate is a powerful agonist of excitation–contraction coupling [4–11]. The underlying mechanisms have been ascribed to a modification of the function of the voltage-sensor in the calcium channels of muscle T-tubules, either directly by interaction with the dihydropyridine (DHP) receptor, causing a shift in the voltage dependence of intramembranous charge movement [8], or possibly indirectly through a primary action on the ryanodine-sensitive Ca^{2+} release channel followed by an allosteric interaction between the release channel and the voltage sensor [9–11].

There is ample evidence from electrophysiological studies [12] and measurements of intracellular calcium [13] that a DHP-sensitive and voltage-gated L-type calcium channel [14], similar to that in muscle cells, is present in the mouse β -cell plasma membrane and that it has a central role in mediating glucose-induced Ca^{2+} inflow and elevation of the intracellular Ca^{2+} activity in β -cells [15]. Inhibition of the β -cell L-type Ca^{2+} channels by nifedipine showed that the perchlorate-potentiating effect on glucose-induced insulin secretion was more sensitive to the DHP blocker than the basic glucose stimulation as such, suggesting that perchlorate may interfere directly with this Ca^{2+} channel [16]. Perchlorate has not yet been shown to affect parts

of the secretory signal-transduction system in the β -cells other than Ca^{2+} handling. Thus, it does not affect the rate of glucose oxidation [1] or glycolysis [J. Sehlín, unpublished work], level of cyclic AMP, $^{36}\text{Cl}^-$ fluxes, $^{86}\text{Rb}^+$ fluxes (K^+ marker) or intracellular pH [17].

To investigate further the mechanisms behind the amplifying effects of perchlorate on β -cell secretory regulation, we have analysed the effects of ClO_4^- and the L-type calcium channel agonist, Bay K 8644, on the dynamics of glucose-stimulated intracellular Ca^{2+} and insulin release in mouse β -cells.

MATERIALS AND METHODS

Animals and isolation of islets

Non-inbred, 7–8 month old, female *ob/ob* mice (Umeå-*ob/ob*) were used throughout. Pancreatic islets from these animals contain an exceptionally high proportion of β -cells (> 90 %; see [18]). Although the mice from this breeding stock are metabolically abnormal with mild hyperglycaemia, which may be due to a peripheral insulin resistance and hyperphagia [19,20], their islets show normal regulation of insulin secretion *in vitro* [21–23]. The high proportion of β -cells in these *ob/ob*-mouse islets makes it highly probable that the present data on isolated islet cells are representative of this cell type. The mice were fasted overnight to normalize their blood sugar [24] and to facilitate the isolation of pancreatic islets. The pancreata were digested with collagenase to isolate individual islets [25]. The medium used was a Krebs-Ringer medium (KRH) with the following salt composition (mM): NaCl, 130; KCl, 4.7; KH_2PO_4 , 1.2; MgSO_4 , 1.2; and CaCl_2 , 2.56. BSA at 10 mg/ml and 3 mM D-glucose were added. The medium was buffered with 20 mM Hepes and NaOH to a final pH of 7.4 and the gas phase was the ambient air. In the isolation of islet cells and in incubations and microperfusions

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytoplasmic Ca^{2+} concentration; KRH, Krebs-Ringer medium; DHP, dihydropyridine.

* To whom correspondence should be addressed.

of islets or cells, the KRH medium contained 1 mg/ml BSA, if not otherwise stated.

Isolation and culture of islet cells

Islets for measurement of cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were dispersed to single β -cells in a Ca^{2+} -free KRH medium supplemented with 1 mM EGTA and 1 mg/ml DNase [25]. The cell suspension was placed on top of a 4% (w/v) BSA column and centrifuged at room temperature for 5 min at 50 *g*. The sediment was then immediately resuspended in KRH medium and portions of the cell suspension were placed on cover glasses, which had been coated with polylysine (0.01% w/v) and allowed to attach for 15 min. The cells were then cultured for 1–2 days in 3 ml of culture medium RPMI 1640 supplemented with 10% (w/v) heat-inactivated fetal calf serum, 11.1 mM glucose, 20 mM Hepes, 2 mM L-glutamine, 60 $\mu\text{g}/\text{ml}$ garamycin and 60 $\mu\text{g}/\text{ml}$ benzylpenicillin.

Measurement of cytoplasmic Ca^{2+}

Cells were loaded with 1 μM fura 2/AM during 40 min of incubation at 37 °C in KRH containing 3 mM D-glucose and 1 mg/ml BSA. The cover glasses with the attached cells were rinsed (KRH) to remove extracellular fura 2 and placed at the bottom of an open micropertusion system [26] with both the inflow and outflow of perfusate driven by a peristaltic pump (Ismatec micropump) at a flow rate of 0.6 ml/min. The perfusion chamber (170 μl) was placed on the stage of an inverted microscope (Nikon Diaphot-TMD) and kept at 37 °C in a thermostated compartment. Individual, non-aggregated cells were selected for measurement. The time for new medium to reach the chamber after switching the valve was determined in each experiment (average 165 s) and has been corrected for in Figures 1–5, so that time zero represents the arrival time of new medium to the chamber. Five seconds after switching from 3 to 20 mM glucose, the glucose concentration in the chamber had reached 14.6 ± 1.2 mM, and after 10 s, 18.6 ± 0.3 mM glucose. Within 15 s the glucose concentration had reached 20 mM ($n = 5$; results not shown). Samples of the medium were collected from the centre of the chamber and close to the bottom, where the analysed cells are located. For measurement of $[\text{Ca}^{2+}]_i$, fura 2 was excited alternately at 340 and 380 nm with band-pass filters in front of a 100 W xenon lamp. The resulting emitted fluorescence was measured with a photomultiplier at 510 nm. The time interval between successive cycles of 340/380 nm excitations was 0.77 s. The basal perfusion medium was KRH containing 3 mM glucose. All cells were initially pre-perfused for 15 min with registration during the last 3 min. After changing the medium, they were then perfused for another 30 min with different test agents. In some experiments, another change of medium was done at 15 min (see legends to Figures). Control and test experiments were run during the same time-period. The apparent concentration of cytoplasmic Ca^{2+} was calculated from the 340/380 nm ratio and calibration was performed using the equation described of Grynkiewicz et al. [27], with a K_d of 224 nM. For clarity, all graphs of $[\text{Ca}^{2+}]_i$ show both the 340/380 nm ratio and the calculated $[\text{Ca}^{2+}]_i$.

Measurement of insulin release

The perfusion of isolated islets was performed essentially as previously described [28]. Collagenase-isolated islets (60 per pancreas) were subjected to a preliminary static incubation for 60 min at 37 °C in KRH containing 3 mM glucose (basal medium) and then distributed into two identical flow-chambers

operated in parallel; each chamber contained 30 islets. The flow rate during the perfusion was 1 ml/min and the temperature was maintained at 37 °C by keeping the whole perfusion apparatus in a temperature-controlled compartment. After a pre-perfusion for 30 min with basal medium, the glucose concentration was changed to either 20 mM glucose alone or 20 mM glucose plus 12 mM ClO_4^- or 1.0 μM Bay K 8644. Time zero represents the arrival of new medium to the chamber. Insulin was measured by radioimmunoassay [29] using ^{125}I -labelled porcine insulin and mouse insulin as standards.

Statistics

Statistical significance was evaluated by using the two-tailed Student's *t*-test for unpaired data on calculated $[\text{Ca}^{2+}]_i$ and for paired data on insulin secretion. In the statistical process, correction was made for difference in variance between groups [30]. When comparisons of average calcium levels over specified time-periods (see Results section) were made, the average calcium level was calculated as the mean of all registered fura 2 values (every 0.77 s) during that period. Results are expressed as mean $\pm$ S.E.M.

Chemicals

S(-)-Bay K 8644 [methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate] from Research Biochem. Int. (Natick, MA, U.S.A.) was dissolved in ethanol to a stock solution of 10 mM and then diluted in KRH to give the desired drug concentrations. Sodium perchlorate (monohydrate) was obtained from Merck (Darmstadt, Germany). Hepes was from Boeringer (Mannheim, Germany). Poly-L-lysine, EGTA, DNase I and collagenase type I were all from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and BSA (fraction V) from Miles (Slough, U.K.). Tissue-culture medium RPMI 1640 and L-glutamine were from Flow Laboratories Limited (Irvine, Ayrshire, Scotland, U.K.) and fetal calf serum from Labkemi AB (Stockholm, Sweden). Benzylpenicillin was from Astra AB (Södertälje, Sweden) and garamycin from Schering Co. (Kenilworth, NJ, U.S.A.). Fura 2/AM and fura were from Molecular Probes Inc. (Eugene, OR, U.S.A.) and porcine ^{125}I -insulin was from Euro-Diagnostica AB (Malmö, Sweden).

RESULTS

Effects of glucose on $[\text{Ca}^{2+}]_i$

After pre-perfusion for 15 min in 3 mM D-glucose, the effect of 20 mM D-glucose on the $[\text{Ca}^{2+}]_i$ in single β -cells was measured (Figure 1). From a baseline, the β -cells first responded to glucose with a brief drop in $[\text{Ca}^{2+}]_i$, averaging 40% (from 47 ± 3 to 28 ± 3 nM; $P < 0.001$). The suppression was followed by a rather consistent, transient peak in $[\text{Ca}^{2+}]_i$ and then a phase of irregular oscillations. The average response from 26 cells exposed to 20 mM glucose alone is shown in the Figure. After the initial slight decrease in $[\text{Ca}^{2+}]_i$, seven of the cells showed a monophasic peak increase in $[\text{Ca}^{2+}]_i$ with a rapid decline to a flat, lower level, which was maintained throughout the rest of the experiment. In 19 cells, after a marked peak increase there was a variety of oscillatory patterns. Except for the large variability in the pattern of $[\text{Ca}^{2+}]_i$ after the initial peak increase, there was also a large variation in the length of the lag-time, counted from the time of glucose entry into the flow chamber to the start of the first peak increase in $[\text{Ca}^{2+}]_i$. To compensate for the variation in this lag-time, and thus make the average curves more intelligible, all individual curves giving rise to the average representations in

Figure 1 Effect of 12 mM perchlorate on glucose-stimulated $[Ca^{2+}]_i$

After 15 min of pre-perfusion with basal medium containing 3 mM glucose (fura 2 signal was registered during the last 3 min), the glucose concentration was raised to 20 mM with or without 12 mM ClO_4^- and the perfusion continued for 30 min. The curves represent mean values $\pm$ S.E.M. (error bars) for 26 (glucose alone) or 28 experiments (glucose plus ClO_4^-). Each experiment is equivalent to one individual cell. The individual curves have been normalized to the start of the peak increase in $[Ca^{2+}]_i$, as described in the Results section. 'G' denotes D-glucose.

Figure 2 Effects of 12 mM perchlorate introduced during continuous glucose-stimulated $[Ca^{2+}]_i$

After perfusion as described in Figure 1, 12 mM perchlorate was added at 15 min. The curve represents mean values $\pm$ S.E.M. (error bars) for 13 experiments (individual cells). The vertical lines surrounding 15 min indicate the temporal displacement of the normalized individual curves (see Figure 1). 'G' denotes D-glucose.

Figures 1–3 and Figure 5 have been adjusted to the average lag-time for each experimental group. This has been done by adjusting the time-scale for individual recordings, so that the start of the first peak increase in $[Ca^{2+}]_i$ in each recording was normalized to the end-point of the average lag-time. However, all statistical analyses have, naturally, been made using the uncorrected time-scales for the individual experiments. The lag-time in perfusions with glucose alone had a range of 1.8–8.9 min with an average of 5.0 ± 0.4 min. The initial average peak height after glucose stimulation (20 mM) was 2.12 ± 0.03 (from recording of the 340/380 nm fluorescence excitation ratio) or, as calculated from the fluorescence ratio according to Grynkiewicz [27], 334 ± 81 nM

$[Ca^{2+}]_i$. After the initial peak, the average $[Ca^{2+}]_i$ level during the period from 10 to 30 min was 132 ± 13 nM.

Effects of perchlorate on glucose-stimulated $[Ca^{2+}]_i$

When 20 mM D-glucose and 12 mM ClO_4^- were added together (Figure 1), the average lag-time before an increase in $[Ca^{2+}]_i$ was shortened by 44 % (2.9 ± 0.4 and 5.0 ± 0.4 min respectively; $P < 0.001$). The lag-time in perfusions with glucose plus ClO_4^- had a range of 0.4–8.3 min. The distributions of the lag-times in the two experimental groups were not different from the normal distribution. The slight drop in $[Ca^{2+}]_i$ was not markedly affected by perchlorate and the major reduction in the lag-time therefore applied to the initial period at baseline level (Figure 1). Perchlorate did not change the peak height, but the average $[Ca^{2+}]_i$ level after the peak was significantly elevated by the anion (207 ± 21 and 132 ± 13 nM Ca^{2+} with and without perchlorate respectively; $P < 0.005$).

Exposure to 12 mM perchlorate 15 min after the addition of 20 mM glucose (Figure 2) significantly increased the average $[Ca^{2+}]_i$ level, as compared with the control (same data as in Figure 1) containing 20 mM glucose alone during the period from 15–30 min (215 ± 20 and 132 ± 13 nM Ca^{2+} , average, respectively; $P < 0.005$). In the individual experiments, the amplifying effects of perchlorate differed. In some cells, already showing a glucose-induced continuous oscillatory calcium level when exposed to perchlorate, there was an increase in the oscillation frequency. In other cells, without oscillations before the addition of perchlorate, either oscillations started and/or there was an increase in the $[Ca^{2+}]_i$ level. The first 15 min of glucose exposure, before ClO_4^- was added, is identical with the experiments with glucose alone, as shown in Figure 1. These experimental data could therefore be pooled. Also, when compared with the pooled data on lag-time (4.5 ± 0.3 min), perchlorate (12 mM) showed a statistically significant shortening of the lag-time ($P < 0.005$).

Figure 3 shows that the addition of 12 mM ClO_4^- in the

Figure 3 Effect of 12 mM perchlorate on $[Ca^{2+}]_i$ at a non-stimulatory glucose level

Perfusion was performed as described in Figure 1. The curves represent mean values $\pm$ S.E.M. (error bars shown when larger than line) for nine experiments (individual cells). The individual curves have been normalized as in Figure 1. 'G' denotes D-glucose.

presence of 3 mM glucose did not change the $[Ca^{2+}]_i$, nor did this pretreatment with perchlorate markedly influence the subsequent stimulation by 20 mM D-glucose alone (peak height 405 ± 43 nM Ca^{2+}). That the lag-time (3.1 ± 0.3 min) was shorter than that shown for the control in Figure 1, might be due to ClO_4^- initially remaining around, and/or in, the β -cells after switching to 20 mM glucose.

The stimulatory effects of 20 mM glucose alone or 20 mM glucose plus 12 mM ClO_4^- on the β -cell calcium level during the 30 min of microperfusion were completely suppressed to baseline level by the omission of extracellular Ca^{2+} ($n = 5$ in both cases; results not shown). Addition of 1 mM EGTA in combination with the Ca^{2+} omission resulted in a slight reduction in $[Ca^{2+}]_i$ below the initial baseline, and this remained throughout the experiments ($n = 5$; results not shown).

Effects of perchlorate on K^+ -stimulated $[Ca^{2+}]_i$

The effect of depolarizing the β -cells with K^+ on $[Ca^{2+}]_i$ in the presence of 3 mM D-glucose is shown in Figure 4. The response to 20 mM K^+ was a prompt, biphasic rise in $[Ca^{2+}]_i$, without any initial decrease in the Ca^{2+} activity like that observed for glucose. The short lag-time was not significantly changed by ClO_4^- [18 ± 2 s (control) and 16 ± 2 s (plus 12 mM ClO_4^-)] and the average initial peak height was only slightly enhanced by ClO_4^- . ClO_4^- amplified the K^+ -stimulated $[Ca^{2+}]_i$, as measured during the 5–10 min period, which in most cells represents the period of decline from peak $[Ca^{2+}]_i$ to a more stable level [241 ± 13 (control) and 307 ± 18 nM Ca^{2+} (plus ClO_4^-); $P < 0.01$]. The data suggest that ClO_4^- counteracts the spontaneous decline from the maximum K^+ -stimulated Ca^{2+} level. Exposing the β -cells to 20 mM K^+ in the absence of glucose, as compared with the presence of 3 mM glucose, resulted in a prolonged lag-time before activation, and that lag-time was reduced by 12 mM ClO_4^- [42 ± 4 s (20 mM K^+ , no glucose) compared with 18 ± 2 s (20 mM K^+ , 3 mM glucose; $P < 0.001$) or 22 ± 1 s (20 mM K^+ and 12 mM ClO_4^- , no glucose, $P < 0.005$)]. Neither the initial peak height nor the subsequent phase of K^+ -stimulated calcium level were significantly changed by the anion in the absence of glucose ($n = 7$, results not shown).

Effects of Bay K 8644 on $[Ca^{2+}]_i$

Our previous results have indicated that perchlorate may interact

Figure 4 Effect of 12 mM perchlorate on K^+ -stimulated $[Ca^{2+}]_i$

Perfusion was performed as in Figure 1. Curves represent mean values $\pm$ S.E.M. (error bars) for 17 experiments (individual cells) (K^+ alone) or 19 experiments (K^+ plus perchlorate). '3 G' refers to 3 mM D-glucose.

with sites in the β -cell close to the DHP-sensitive Ca^{2+} channel [16]. To investigate whether the various aspects of the perchlorate effect on intracellular calcium could be mediated by the L-type calcium channel, we compared the perchlorate effects with those of the well-established DHP calcium channel agonist, Bay K 8644 (Figure 5). Bay K 8644 (0.01, 0.1 or 1.0 μ M) reduced the average lag-time of glucose-induced increase in $[Ca^{2+}]_i$ from 5.0 ± 0.4 to 3.1 ± 0.3 min ($P < 0.001$). The effect of Bay K 8644 showed no concentration dependence in this concentration range, which made it convenient to pool the data for the statistical analysis. The slight drop in $[Ca^{2+}]_i$ was not markedly affected by Bay K 8644. The initial peak stimulation reached 573 ± 39 nM Ca^{2+} , but the increase above control was not statistically significant. The subsequent phase during min 10–30 was enhanced from the control level of 132 ± 13 (same data as in Figure 1) in the absence of the drug to 197 ± 26 nM Ca^{2+} with the agonist present ($P < 0.05$). When 10 μ M Bay K 8644 was added, the average lag-time was markedly prolonged to 9.5 ± 2.9 min ($n = 4$) and the cells expressed only a transient peak increase and then rapidly returned to baseline level, where the $[Ca^{2+}]_i$ signal was kept for the remaining part of the experiments (results not shown). That this calcium channel agonist at higher concentrations can turn into an inhibitor of the pancreatic β -cells has been shown [31].

Effects of ClO_4^- or Bay K 8644 on insulin secretion

Since perchlorate and Bay K 8644 showed almost identical effects on glucose-stimulated $[Ca^{2+}]_i$, it was of interest to in-

Figure 5 Effect of Bay K 8644 on glucose-stimulated $[Ca^{2+}]_i$

Perfusions were performed as in Figure 1. The control curve is identical with that in Figure 2 and the curve representing Bay K 8644 represents pooled data (30 experiments) from experiments with 0.01 μ M (5 experiments), 0.1 μ M (17 experiments) and 1 μ M (8 experiments) of the drug. Curves denote mean values $\pm$ S.E.M. (error bars). The individual curves have been normalized as in Figure 1. 'G' denotes D-glucose.

Figure 6 Effect of 12 mM perchlorate on the dynamics of glucose-induced insulin release

After pre-perfusion of islets for 30 min in 3 mM glucose, the medium was changed to 20 mM glucose with or without 12 mM perchlorate. Symbols denote mean values $\pm$ S.E.M. (error bars given when larger than symbol). '3 G' denotes 3 mM D-glucose. The inset shows the initial fractions at high glucose concentration with higher resolution on both axes.

investigate their effects on the dynamics of glucose-stimulated insulin secretion (Figure 6). Intact pancreatic islets were chosen for these experiments in order to obtain a complete secretory response. After switching from 3 to 20 mM glucose, the perfusate was fractionated at short intervals (15 s). Control and test islets in each experiment were strictly taken from the same animal and the islets were perfused simultaneously in two parallel flow chambers to obtain high experimental precision. ClO_4^- (12 mM) combined with 20 mM glucose clearly shortened the lag-time for stimulated insulin secretion ($P < 0.001$). When glucose was added

alone, significant stimulation of insulin release above baseline occurred after 75 s ($P < 0.025$ for the 75–90 s period), and when ClO_4^- was combined with glucose, after 15 s ($P < 0.05$ for the period 15–30 s). The augmentation of glucose-stimulated insulin secretion by perchlorate remained throughout the experiment (30 min), i.e. during both the first and second phases of insulin release. Similar results, as regards the effects on lag-time and first and second phase insulin secretion, were obtained with the combination of 20 mM glucose and 1.0 μ M Bay K 8644 (Figure 7). In this series of experiments, significant stimulation of insulin

Figure 7 Effect of 1 μ M Bay K 8644 on the dynamics of glucose-induced insulin release

Islets were perfused as described in Figure 6, except that the effect of Bay K 8644 was studied. Symbols denote mean values $\pm$ S.E.M. (error bars shown when larger than symbol). '3 G' denotes 3 mM D-glucose. The inset shows the initial fractions at high glucose concentration with higher resolution on both axes.

release above baseline occurred for glucose alone after 75 s ($P < 0.02$ for the period 75–90 s) and for Bay K 8644 combined with glucose after 30 s ($P < 0.02$ for the period 30–45 s). The difference in lag-time was statistically significant ($P < 0.001$). The first and second phases of insulin release were enhanced by this calcium-channel agonist in a way quite similar to that exerted by perchlorate.

DISCUSSION

The present results confirm previous findings that the chaotropic anion, perchlorate, is an amplifier of stimulated insulin secretion [1] and add new data as to the cellular mechanisms involved. Perchlorate markedly amplified both the first and second phases of glucose-induced insulin release. Previous results have shown that perchlorate, strictly, does not affect basal insulin release in the absence of a primary stimulus [1] and that the amplifying effect applies to both nutrient secretagogues, like glucose [1] or leucine [Sehlin, unpublished data], and non-nutrient stimuli, like elevated extracellular K^+ concentration [1] or sulphonylurea [Sehlin, unpublished data]. The fact that perchlorate can amplify the primary stimulation by these different stimuli, which utilize partly different signal pathways, and that the anion has, so far, been shown to lack effects on secretory signal mechanisms other than Ca^{2+} (see Introduction), strengthen the view that perchlorate interacts with the calcium-handling system of the pancreatic β -cell. This is further strengthened by the present finding that perchlorate amplified the K^+ -induced level of intracellular calcium. It seems likely that the agonist effect of perchlorate on stimulus–secretion coupling in the β -cells, like the amplification of excitation–contraction coupling in skeletal muscle caused by the anion [4–11], may be caused by an interaction with voltage-gated calcium uptake.

A distinct effect of perchlorate in the present experiments was the activation of the early dynamics of insulin secretion. It is well established that pancreatic islets stimulated by glucose display an

initial silent lag-phase of about 1–2 min before stimulated insulin release commences (see [32]), but the precise underlying mechanisms are obscure. The present data show that perchlorate strongly shortened this lag-time, causing an earlier onset of first-phase insulin release, which was also markedly amplified. The results on the dynamic development of the stimulated intracellular calcium level showed a general elevation of the average stimulated calcium level, as indicated by fura 2. This is similar to the effects of perchlorate on secretion, and the elevated calcium signal would, according to general theory, mediate the general amplification of the stimulated insulin release. Similar to the effect on stimulated secretion, perchlorate markedly shortened the lag-time for stimulated $[Ca^{2+}]_i$. It seems very likely that this effect is responsible for the shortened lag-time in the insulin release. It is also evident, however, that the average lag-time for glucose-induced insulin release in islets is shorter than the lag-time for stimulated $[Ca^{2+}]_i$ in isolated β -cells. This seems to be connected with the β -cell signal transduction for glucose, since the β -cell preparations used responded promptly to high K^+ with an increase in $[Ca^{2+}]_i$. In intact islets the β -cells are coupled to each other and to other endocrine cells in an integrated functional system. It is therefore probably not realistic to expect precisely the same reactivity in single β -cells and intact islets. It should be noted that the relative effects of perchlorate on the lag-times of stimulated Ca^{2+} and secretion are rather similar in magnitude.

The effect of perchlorate on $[Ca^{2+}]_i$ was abolished by extracellular Ca^{2+} deficiency, indicating that it concerns amplification of the inflow of extracellular calcium. However, even if we exclude parts of the β -cell stimulus–secretion coupling other than calcium inflow (see above), perchlorate could interact with calcium uptake through different specific mechanisms. We will analyse two such possible mechanisms. Chaotropic anions, such as perchlorate, can adsorb on phospholipid membranes and induce a negative electrostatic potential at the membrane surface [33], which could shift the voltage dependence of the Ca^{2+} channels and other channels to more negative potentials. How-

ever, as discussed in detail elsewhere [1], at the relatively low concentration of perchlorate used, and considering previous results on frog muscle [4,5], it is unlikely that the present effects of perchlorate on the β -cells are caused by a general electrostatic change of the plasma membrane surface.

Another plausible mechanism for the perchlorate effects on $[Ca^{2+}]_i$ described here is an effect on the calcium-channel voltage sensor (DHP receptor), in analogy with the traditional views on excitation-contraction coupling in skeletal muscle (see [9]). To test whether a direct agonist effect on the voltage sensor could produce the observed pattern of changes in stimulated intracellular calcium level and insulin secretion, we used an established L-type calcium-channel agonist, Bay K 8644 [34], under experimental conditions identical with those used in the perchlorate experiments. Indeed, all facets of the perchlorate effects could be reproduced with this drug, i.e. an elevation of the average glucose-stimulated $[Ca^{2+}]_i$ and insulin secretion and a marked shortening of the lag-times of both stimulated intracellular calcium and insulin release. Bay K 8644 is known to interact with the DHP receptor by increasing the mean open time and opening probability of the channels in a variety of cells, including cardiac cells [35,36], as well as pancreatic β -cells [15]. The drug does not change the membrane potential of the resting β -cell [37], i.e. Bay K 8644 acts on the Ca^{2+} channel in its open state, and it fails to affect basal insulin secretion at non-stimulatory glucose concentrations [31] but amplifies stimulated insulin secretion ([31] and present results). Provided that Bay K 8644 selectively acts on the voltage sensor of L-type calcium channels, the closely similar effects of this agonist and perchlorate would suggest that perchlorate also acts directly on the voltage sensor. Furthermore, these results suggest that both the shortening of lag-time and amplification of the stimulated phases of $[Ca^{2+}]_i$ can be accommodated within the same basic mechanism of action of perchlorate. Glucose-stimulated insulin secretion shows anion selectivity, following the Hofmeister series [2], with perchlorate showing the strongest effect. Although the physiological relevance of this anion selectivity is unclear, our results point to the possibility that the L-type calcium channel in β -cells is under the influence of the surrounding anion composition. Whether naturally occurring anionic metabolites or other cellular anion products can influence the stimulus-secretion coupling process in β -cells by directly modifying the calcium channel remains to be determined.

Bay K 8644 is believed to affect the α -1 subunit of the DHP-sensitive Ca^{2+} channel [38]. This causes stabilization of the channel in the open state and thereby enhances Ca^{2+} influx [39]. Chaotropic anions, at least at high concentrations (> 100 mM), are thought to interact with macromolecules and membranous materials, mainly indirectly, by affecting the interphase between the aqueous solvent and the dissolved molecules [40]. The present data concerning the effects of low concentrations of perchlorate do not provide information as to the exact interaction between perchlorate and the voltage sensor, but, as chaotropic anions can bind to the amide dipole in peptides with affinities following the Hofmeister series [41], perchlorate could probably directly interact with the tertiary structure of the calcium channel. It is an intriguing possibility that the anion selectivity of insulin secretion, which also follows the Hofmeister series [2], can be explained by direct interaction between anions and the calcium channel.

This work was supported in part by the Swedish Medical Research Council (grant no. 12X-4756), the Swedish Diabetes Association, the Elsa and Folke Sahlberg Fund, Novo-Nordisk AB and the Medical Faculty at Umeå University.

REFERENCES

- Sehlin, J. (1987) *Biochem. J.* **248**, 109–115
- Sehlin, J. (1987) in *Biophysics of the Pancreatic β -Cell* (Atwater, I., Rojas, E. and Soria, B., eds.), pp. 227–233, Plenum Press, New York
- Ohlsson, L., Lindström, P. and Sehlin, J. (1989) *Acta Physiol. Scand.* **137**, 545–546
- Gomolla, M., Gottschalk, G. and Lüttgau, H. C. (1983) *J. Physiol. (London)* **343**, 197–214
- Lüttgau, H. C., Gottschalk, G., Kóvacs, L. and Fuxreiter, M. (1983) *Biophys. J.* **43**, 247–249
- Csernoch, L., Kóvacs, L. and Szücs, G. (1987) *J. Physiol. (London)* **390**, 213–227
- Huang, C. L.-H. (1987) *J. Physiol. (London)* **384**, 491–509
- Gallant, E. M., Taus, N. S., Fletcher, T. F., Lentz, L. R., Louis, C. F. and Mickelson, J. R. (1993) *Am. J. Physiol.* **264**, C559–C567
- González, A. and Ríos, E. (1993) *J. Gen. Physiol.* **102**, 373–421
- Ma, J., Anderson, K., Shirokov, R., Levis, R., González, A., Karhanek, M., Hosey, M. M., Meissner, G. and Ríos, E. (1993) *J. Gen. Physiol.* **102**, 423–448
- Ríos, E., Karhanek, M., Ma, J. and González, A. (1993) *J. Gen. Physiol.* **102**, 449–481
- Ashcroft, F. M. and Rorsman, P. (1989) *Prog. Biophys. Mol. Biol.* **54**, 87–143
- Hellman, B., Gylfe, E., Grapengiesser, E., Lund, P.-E. and Marcström, A. (1991) in *Nutrient Regulation of Insulin Secretion* (Flatt, P. R., ed.), pp. 213–246, Portland Press, London
- Nowicky, M. C., Fox, A. P. and Tsien, R. W. (1985) *Nature (London)* **316**, 440–443
- Smith, P. A., Rorsman, P. and Ashcroft, F. M. (1989) *Nature (London)* **342**, 550–553
- Larsson-Nyrén, G. and Sehlin, J. (1993) *Biosci. Rep.* **13**, 107–117
- Sehlin, J., Engström, K. G., Frankel, B. J., Larsson-Nyrén, G., Lindström, P., Sandström, P.-E. and Åmark, K. (1994) in *Insulin Secretion and Pancreatic β -Cell Research* (Flatt, P. R. and Lenzen, S., eds.), pp. 195–200, Smith Gordon, London
- Hellman, B. (1965) *Ann. N.Y. Acad. Sci.* **131**, 541–548
- Stauffer, W. and Renold, A. E. (1969) *Am. J. Physiol.* **216**, 98–105
- Stauffer, W., Lambert, A. E., Vechio, D. and Renold, A. E. (1967) *Diabetologia* **3**, 230–237
- Hahn, H.-J., Hellman, B., Lernmark, Å., Sehlin, J. and Täljedal, I.-B. (1974) *J. Biol. Chem.* **249**, 5275–5284
- Hellman, B., Idahl, L.-Å., Lernmark, Å., Sehlin, J. and Täljedal, I.-B. (1974) *Biochem. J.* **138**, 33–45
- Lindström, P. and Sehlin, J. (1983) *Endocrinology* **112**, 1524–1529
- Sandström, P.-E. and Sehlin, J. (1988) *Eur. J. Pharmacol.* **147**, 403–409
- Lernmark, Å. (1974) *Diabetologia* **10**, 431–438
- Grapengiesser, E., Gylfe, E. and Hellman, B. (1989) *Arch. Biochem. Biophys.* **268**, 404–407
- Gryniewicz, G., Poenie, M. and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Lindström, P. and Sehlin, J. (1982) *Biochim. Biophys. Acta* **720**, 400–404
- Heding, L. G. (1966) in *Labelled Proteins in Tracer Studies* (Donato, L., Milhaud, G. and Sirchis, G., eds.), pp. 345–351, Euratom, Brussels
- Davies, O. L. and Goldsmith, P. L. (1980) *Statistical Methods in Research and Production*, 4th edn., pp. 61–62, Longman, London
- Malaisse-Lagae, F., Mathias, P. C. F. and Malaisse, W. J. (1984) *Biochem. Biophys. Res. Commun.* **123**, 1062–1068
- Idahl, L.-Å. (1973) *Diabetologia* **9**, 403–412
- McLaughlin, S., Bruder, A., Chen, S. and Moser, C. (1975) *Biochim. Biophys. Acta* **394**, 304–313
- Schramm, M., Thomas, G., Towart, R. and Franckowiak, G. (1983) *Nature (London)* **303**, 535–537
- Hess, P., Lansman, J. B. and Tsien, R. W. (1984) *Nature (London)* **311**, 538–544
- Kokubun, S. and Reuter, H. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4824–4827
- Lebrun, P. and Atwater, I. (1985) *Biophys. J.* (1985) **48**, 919–930
- Caterall, W. A. and Striessnig, J. (1992) *Trends Pharm. Sci.* **13**, 256–262
- Bechem, M. and Hoffman, H. (1993) *Pflügers Arch.* **424**, 343–353
- Hanstein, W. G. (1979) *J. Solid-Phase Biochem.* **4**, 189–206
- von Hippel, P. H., Peticolas, V., Schack, L. and Karlson, L. (1973) *Biochemistry* **12**, 1256–1264