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Stimulant-Evoked Depolarization and Increase in $[Ca^{2+}]_i$ in Insulin-Secreting Cells Is Dependent on External Na^+

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Summary. The patch-clamp technique and measurements of single cell $[Ca^{2+}]_i$ have been used to investigate the importance of extracellular Na^+ for carbohydrate-induced stimulation of RINm5F insulin-secreting cells. Using patch-clamp whole-cell (current-clamp) recordings the average cellular transmembrane potential was estimated to be -60 ± 1 mV ($n = 83$) and the average basal $[Ca^{2+}]_i$ 102 ± 6 nM ($n = 37$). When challenged with either glucose (2.5–10 mM) or D-glyceraldehyde (10 mM) the cells depolarized, which led to the initiation of Ca^{2+} spike potentials and a sharp rise in $[Ca^{2+}]_i$. Similar effects were also observed with the sulphonylurea compound tolbutamide (0.01–0.1 mM). Both the generation of the spike potentials and the increase in $[Ca^{2+}]_i$ were abolished when Ca^{2+} was removed from the bathing media. When all external Na^+ was replaced with N-methyl-D-glucamine, in the continued presence of either glucose, D-glyceraldehyde or tolbutamide, a membrane repolarization resulted, which terminated Ca^{2+} spike potentials and attenuated the rise in $[Ca^{2+}]_i$. Tetrodotoxin (TTX) (1–2 μM) was also found to both repolarize the membrane and abolish secretagogue-induced rises in $[Ca^{2+}]_i$.

Key Words patch clamp · $[Ca^{2+}]_i$ · Na^+ dependency · RINm5F cell · fura-2 · whole cell

Introduction

Secretion of insulin from pancreatic β-cells of the islets of Langerhans is primarily controlled by the membrane potential (Petersen & Findlay, 1987; Ashcroft, 1988; Petersen, 1988). In intact resting cells open ATP-sensitive potassium (K_{ATP}) channels (Cook & Hales, 1984) dominate the permeability of the plasma membrane (Findlay, Dunne & Petersen, 1985; Rorsman & Trube, 1985). Carbohydrate secretagogues (such as glucose or D-glyceraldehyde) evoke closure of these channels causing membrane depolarization (Ashcroft, Harrison & Ashcroft, 1984; Dunne et al., 1986; Misler et al., 1986). This depolarization is required for the opening of voltage-gated Ca^{2+} channels (Matthews & Sakamoto, 1975; Velasco, Petersen & Petersen, 1988), which leads to an increase in the free intracellular calcium ion concentration ($[Ca^{2+}]_i$), the key intracellular regulator of insulin secretion (Wollheim & Biden, 1987). Closure of a large fraction of K^+ channels alone, however, would not depolarize the cell in the absence of an inward current, required to drive the membrane potential away from the K^+ equilibrium potential (E_K) (Petersen & Findlay, 1987; Cook et al., 1988). Since it has previously been shown that glucose-induced insulin release in β-cells is reduced in media containing low Na^+ (Lambert, Henquin & Orci, 1974; Hales & Milner, 1968*a*) and enhanced under conditions favoring increases in intracellular Na^+ concentrations (Lowe et al., 1976; Hales & Milner, 1968*b*), we have investigated the effects of changes in the $[Na^+]_o$ upon stimulus-secretion coupling in the clonal insulin-secreting cell line RINm5F.

A combination of techniques have been used. We have monitored changes in the transmembrane potential of the cell using the patch-clamp whole-cell (current-clamp) recording configuration and assessed single cell $[Ca^{2+}]_i$ using dual-excitation microfluorimetry with fura-2. Stimulation of RINm5F cells was brought about in one of two ways. Either cells were exposed to the carbohydrate secretagogues glucose (Ribalet, Eddlestone & Ciani, 1988; Dunne et al., 1989*b*) or D-glyceraldehyde (Praz et al., 1983; Dunne et al., 1986), both of which have been shown to evoke the release of insulin from these cells, through the selective closure of ATP-sensitive K^+ channels, alternatively, stimulation was initiated by the sulphonylurea compound tolbutamide, which selectively closes K_{ATP} channels in mouse (Trube, Rorsman & Ohno-Shosaku, 1986), clonal (Sturgess et al., 1985; Dunne, Ilott & Petersen, 1987) and human insulin-secreting cells (Ashcroft et al., 1987).

Methods and Materials

Cell Isolation and Maintenance

All experiments were carried out on the clonal insulin-secreting cell line RINm5F, maintained as previously described (Dunne et al., 1988*b*; 1989*a*).

Media

The standard extracellular Na^+-rich solution, contained (in mM); 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 $CaCl_2$ and 10 HEPES. The pH was set at 7.2 (NaOH). The "Na^+-free" solution used in a number of experiments, contained (in mM); 140 N-methyl-D-glucamine Cl, 4.7 KCl, 1.13 $MgCl_2$, 2.5 $CaCl_2$ and 10 HEPES. The pH was set at 7.2 (KOH). All patch-clamp experiments were carried out using a K^+-rich solution in the recording pipette, containing (in mM); 140 KCl, 10 NaCl, 1.13 $MgCl_2$, 10 HEPES, 1 EGTA and 1 ATP. No $CaCl_2$ was added, and the pH was set at 7.2 (KOH). The osmolality of all solutions was 290 ± 5 mOsm/kg. All experiments were conducted at room temperature 22–25°C.

Patch-Clamp Experiments

Electrophysiological investigations of RINm5F cells were carried out using the patch-clamp whole-cell current-clamp recording configuration (Hamill et al., 1981), with the K^+-rich solution in the recording pipette and the Na^+-rich solution in the bath. Patch-clamp pipettes (Type 101 PB, Ceebee Glass, Denmark) were found to have a final resistance of between 5 and 10 MΩ when filled with the K^+-rich solution. Gigaohm seal formation was made in the continuous flow of the Na^+-rich bathing solution from one of a series of outlet pipes, the other reservoirs containing a variety of test solutions (Dunne, Findlay & Petersen, 1988*a*). Whole-cell recordings of membrane potential (zero-current voltage) were stored on FM tape (Racal 4DS recorder) for subsequent replay and analysis. All records presented are photographs of the trace obtained from a low-resolution pen-recorder (Devices, UK). All data was prefiltered at 100 Hz (low pass).

Measurements of Single Cell $[Ca^{2+}]_i$

RINm5F cells were loaded with fluorescence indicator fura-2 by a 30-min preincubation in the Na^+-rich solution, containing 3 μM fura-2-acetoxymethyl ester at room temperature. Following incubation, cells were transferred to an experimental chamber (volume = 0.15 ml) mounted on the stage of a Nikon Diaphot microscope, as previously described (Yule & Gallacher, 1988). Changes in single cell $[Ca^{2+}]_i$ were measured using dual-excitation microfluorimetry (Grynkiewicz, Poenie & Tsien, 1985), with a Spex (Glen spectra) DM 3000 CM system providing alternative excitation wavelengths of 340 and 380 nm, at not less than 10 Hz. The fluorescence emitted at 505 nm was monitored using a photon counter. $[Ca^{2+}]_i$ was assessed from the ratio (R) of the fluorescence at the two discreet wavelengths according to the formula (Schlegel et al., 1987):

$$[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$$

where K_d = 225 nM (Grynkiewicz et al., 1985), R_{max}, R_{min} and β are constants; 7.9 ± 0.4 (n = 6), 0.49 ± 0.08 (n = 6) and 3.9 ± 0.4 (n = 6), respectively. These constants were determined using the *in situ* calibration procedures described by Schlegel et al. (1987, 1988). All records have been corrected for autofluorescence at each wavelength (determined in unloaded cells) before the ratio was calculated.

Results

The Effects of Glucose, D-Glyceraldehyde and Tolbutamide on the Membrane Potential and $[Ca^{2+}]_i$

Changes in the transmembrane potential of RINm5F cells brought about by either glucose, D-glyceraldehyde or tolbutamide have been monitored using the patch-clamp whole-cell recording configuration. In total, 83 cells were investigated. On average the spontaneous zero-current membrane potential, estimated within seconds of forming the whole cell, was found to be −60 ± 1 mV (n = 83).

The effects of glucose on the membrane potential are shown in Fig. 1 (upper panel). In the absence of carbohydrate, the cell has a stable potential of around −70 mV. When challenged with 2.5 mM glucose, added to the bath solution in contact with the extracellular membrane surface, a slow depolarization of the membrane was initiated. Some 35 sec later this was followed by a sharp, more marked depolarization associated with oscillatory waves of depolarization/repolarization with superimposed spike potentials. These spike potentials are Ca^{2+} action potentials, as originally described by Matthews and Sakamoto (1975). Ca^{2+} spike potentials were seen in 73% (54/74) of all cells tested, which probably reflects heterogeneity in the division cycles of the RINm5F cell population used in our study (Rorsman, Arkhammar & Berggren, 1986). In 45 of these whole cells the period of spike-potential generation was found to last on average 308 ± 32 sec (n = 45) after the initiation of the experiment. The disappearance of these spikes is probably due to run-down of Ca-channels (Byerly & Hagiwara, 1982; Fenwick, Marty & Neher, 1982; Katayama, Hofmann & Trautwein, 1985). In nine other whole cells, spike potentials persisted for more than 800 sec, and one cell appeared to be totally functional some 63 min after forming the whole-cell configuration. It is during the Ca^{2+}-spike period that Ca^{2+} enters the cell, which leads to a marked increase in $[Ca^{2+}]_i$ (Fig. 1, lower panel).

The upper panel of Fig. 1 is typical of 46 separate whole cells that were exposed to glucose, at concentrations between 2.5 and 10 mM (75 times). A quantification of the average change in membrane potential, as well as the average time to peak depo-

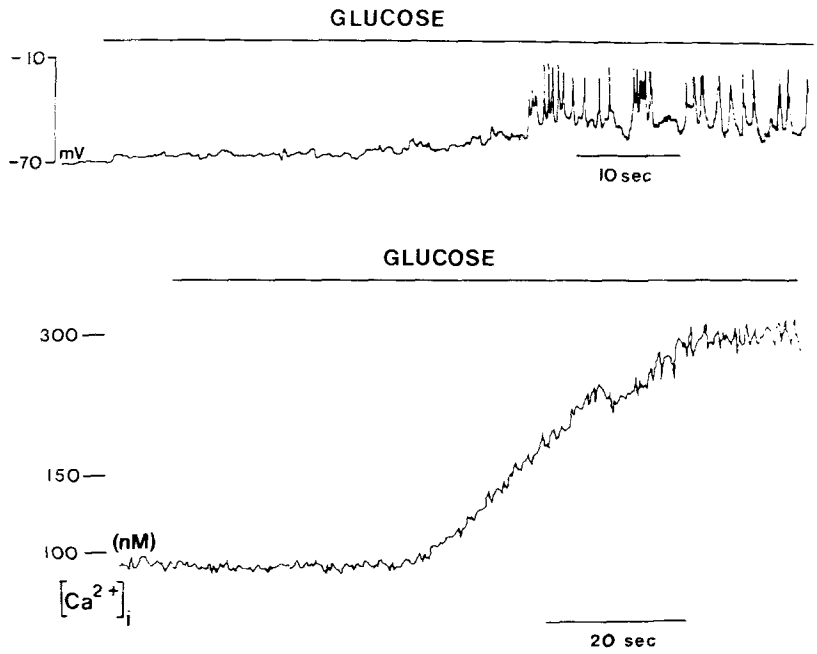


Fig. 1. The effects of glucose (2.5 mM) upon the membrane potential (upper panel) and the free intracellular calcium ion concentration ($[Ca^{2+}]_i$) (lower panel) of individual RINm5F cells. The record shown in the upper panel was obtained using the patch-clamp whole-cell current-clamp mode, and began 40 sec after the whole-cell formation. Measurements of changes in $[Ca^{2+}]_i$, lower panel, were assessed using dual-excitation microfluorimetry with fura-2. The two records came from separate cells

Table 1. The effects of glucose, glyceraldehyde and tolbutamide upon the average (mean ± SEM) change in membrane potential and time to peak depolarization[a]

	Glucose	Glyceraldehyde	Tolbutamide
Total number of whole-cell expts.	46	7	62
Total number of applications	75	13	76
Average depolarization (mV)	23 ± 1	26 ± 3	27 ± 2
Average time-to-peak depolarization (sec)	38 ± 4	11 ± 4	4 ± 0.5

[a] Mean ± SEM values have been determined from the total number of applications of each secretagogue.

Table 2. The effects of glucose and tolbutamide upon the average (mean ± SEM) rise in $[Ca^{2+}]_i$ in the RINm5F cell line[a]

	Glucose	Glyceraldehyde	Tolbutamide
Total number of single cell expts.	21	4	12
Total number of applications	26	4	15
Average peak $[Ca^{2+}]_i$ (nM)	269 ± 10	189 ± 13	364 ± 9

[a] Mean ± SEM values have been determined from the total number of applications of each secretagogue.

larization for these cells has been presented in Table 1. Table 2 summarizes the effects of glucose upon changes in $[Ca^{2+}]_i$.

Only five applications of glucose (5/75) had no effect on either the membrane potential, spike potentials or $[Ca^{2+}]_i$. Changing the glucose concentration from 2.5 to 10 mM, and vice-versa, had no significant influence on the carbohydrate-evoked depolarization, frequency of action-potential generation (n = 3 whole cells, 5/5 applications) or change in $[Ca^{2+}]_i$ (n = 8). In the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$ = $<10^{-9}$ M), both the generation of Ca^{2+} spike potentials (n = 9, 19/19) and the glucose-induced rise in $[Ca^{2+}]_i$ (n = 8 cells) were abolished (Fig. 2). Similar effects to those described for glucose were also seen with D-glyceraldehyde (10 mM) (Tables 1 and 2) and with the sulphonylurea tolbutamide (0.01–0.1 mM) (Tables 1 and 2, and Fig. 3).

Na^+ Dependency of the Effects of Glucose, D-Glyceraldehyde and Tolbutamide on Membrane Potential and $[Ca^{2+}]_i$

Figure 4*A* shows that 5 mM glucose initiates the depolarization of a single RINm5F cell, bringing about the generation of Ca^{2+} spike potentials (upper panel), and an increase in $[Ca^{2+}]_i$ (lower panel). However, if, in the continued presence of glucose, extracellular Na^+ ions are removed, by replacement with 140 mM N-methyl-D-glucamine (NMDG), the cell undergoes a sharp repolarization, which termi-

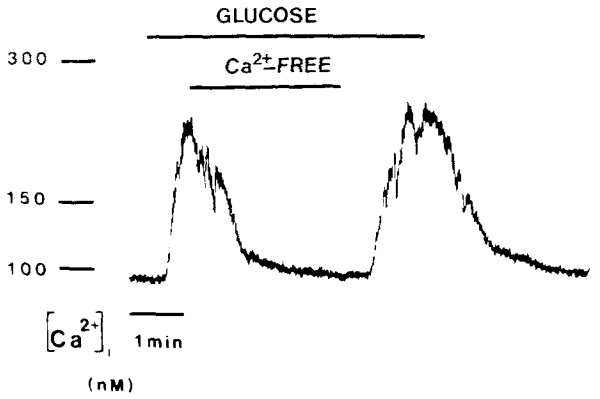
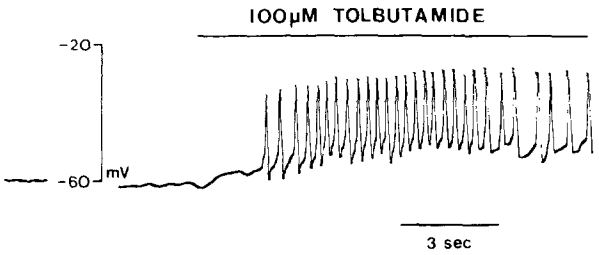


Fig. 2. The glucose (2.5 mM)-induced rise in $[Ca^{2+}]_i$ was dependent upon the extracellular concentration of Ca^{2+}. At the point indicated ("Ca^{2+}-free") Ca^{2+} was removed from the bathing media, by chelation with EGTA, in the continued presence of glucose. This resulted in a marked attenuation of the glucose-induced rise in $[Ca^{2+}]_i$



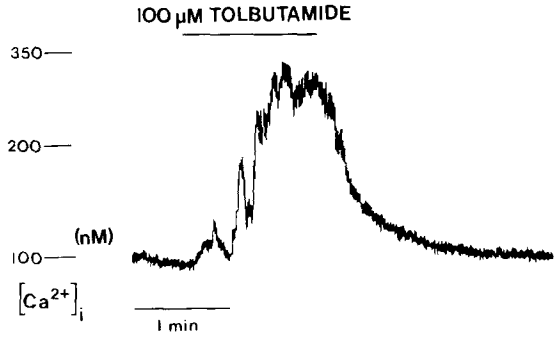


Fig. 3. The action of the sulphonylurea drug tolbutamide (100 μM) upon the membrane potential (upper panel) and $[Ca^{2+}]_i$ (lower panel) of individual RINm5F cells. The two records came from separate RINm5F cells, the upper panel beginning 25 sec after starting the intracellular recording

nates the Ca^{2+} spike potentials (upper panel) and attenuates the glucose-induced rise in $[Ca^{2+}]_i$ (lower panel). When Na^+ ions are re-admitted in the presence of glucose, a renewed depolarization results, reintroducing spike potentials. These records are typical of 6 (upper panel) and 4 (lower panel) other cells, respectively. Similar effects were also found for both tolbutamide, Fig. 4*B* (upper panel; $n = 7$ cells (12/12 applications), lower panel; $n = 5$ separate cells), and glyceraldehyde ($n = 7$ cells (12/12 applications)).

The sensitivity of the effects of glucose and tolbutamide to tetrodotoxin (TTX) (1–2 μM) were also investigated. TTX was added to 21 separate whole cells in the continued presence of glucose a total of 29 times. Seven of the 29 additions caused a clear repolarization of the membrane, of between 5 and 10 mV, which terminated the associated Ca^{2+} spike potentials (Fig. 5). Fifteen of the 29 applications had no significant effect upon the membrane potential, but did abolish the Ca^{2+}-spikes, whereas 7/29 additions had no obvious actions upon either the membrane potential or Ca^{2+} spike potentials.

In the continued presence of tolbutamide the effects of TTX were once again mixed. In all TTX (1–2 μM) was added 35 times during 26 separate whole-cell recordings. Twenty-three of the 35 applications repolarized the membrane (Fig. 6*A*), 5 of the additions abolished Ca^{2+} spike potentials, without any significant effect on the membrane potential, (Fig. 6*B*), and 7 attempts had no effects. Figure 7 shows that TTX attenuates the tolbutamide-induced rise in $[Ca^{2+}]_i$, a result typical of 3 separate cells. Amiloride (1–10 μM), an inhibitor of the Na^+/H^+ exchanger, had no effect on either the glucose- or tolbutamide-induced change in membrane potential, Ca^{2+}-spike generation ($n = 6/9$ applications to 5 separate cells) and rise in $[Ca^{2+}]_i$ ($n = 5$ cells).

Discussion

In the present study we have used a combination of patch-clamp whole-cell recording and single cell $[Ca^{2+}]_i$ measurements to investigate the involvement of external Na^+ ions in stimulus-secretion coupling in the clonal insulinoma cell line RINm5F.

The RINm5F cell line has been a useful model system for investigations of the regulation of insulin secretion. RINm5F cells had, at one stage, been thought not to secrete insulin readily in response to glucose, probably due to a regulatory dysfunction (Halban, Praz & Wollheim, Praz et al., 1983), but recently Ribalet et al. (1988) have shown that the cells respond to glucose stimulation by a mechanism involving the specific closure of K^+ channels. This finding has been confirmed in our experiments (Fig. 1). Glucose, at lower thresholds than required in normal β-cells (Hedeskov, 1980), depolarizes the

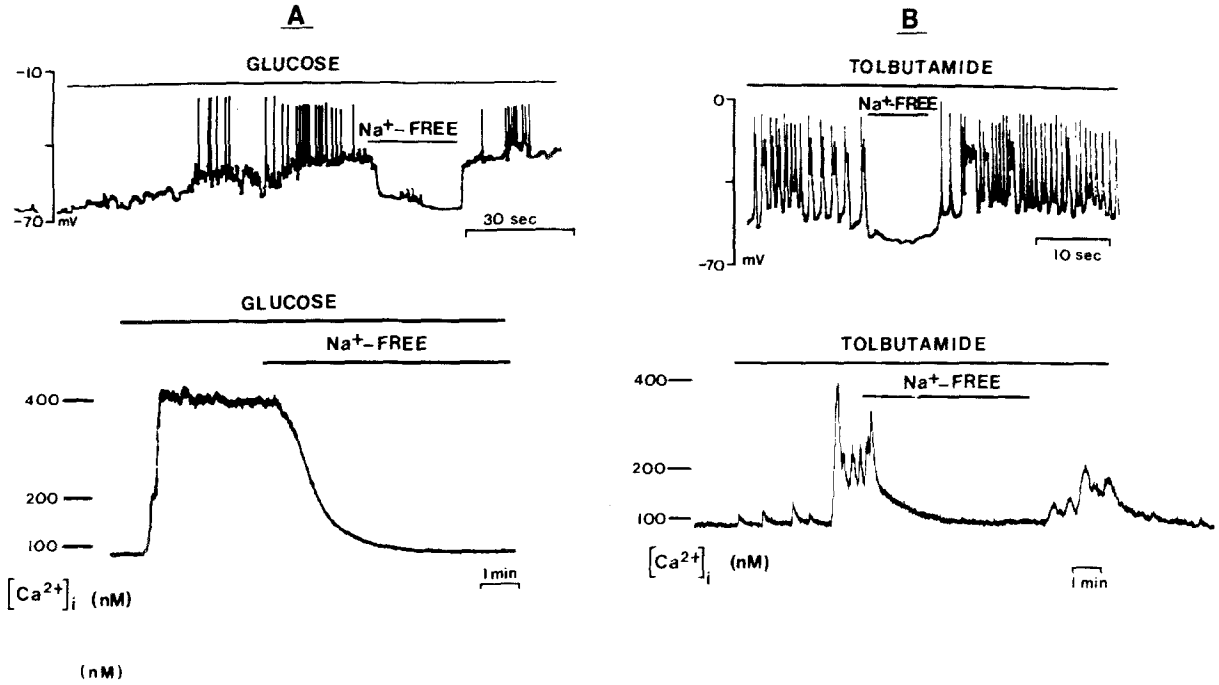


Fig. 4. The effects of (*A*) glucose and (*B*) tolbutamide upon the membrane potential (upper panels) and $[Ca^{2+}]_i$ (lower panels) was dependent upon extracellular Na^+. At the points indicated ("Na^+-free") Na^+ was removed from the bathing solution by replacement with 140 mM N-methyl-D-glucamine; causing a repolization of the membrane, the termination of Ca^{2+} spike potentials and an attenuation of the secretagogue-induced rise in $[Ca^{2+}]_i$. All four experiments came from separate RINm5F cells, the upper panels of (*A*) and (*B*) beginning 10 and 20 sec after starting the respective experiments

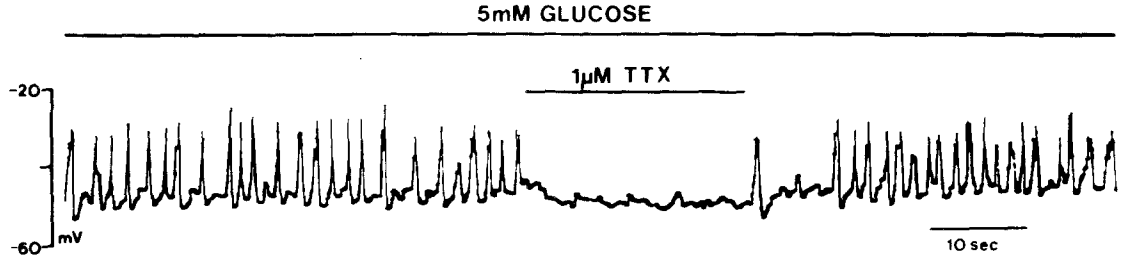


Fig. 5. The action of tetrodotoxin (TTX) (1 μM) upon the glucose-induced electrical activity of an individual RINm5F cell. TTX was able to evoke a reversible repolarization of the membrane, terminating the generation of Ca^{2+} spike potentials

membrane, initiates the firing of Ca^{2+} spike potentials and increases $[Ca^{2+}]_i$. The actions of glucose can be mimicked both by glyceraldehyde (Table 1) and by the sulphonylurea compound tolbutamide (Tables 1 and 2, and Fig. 3).

The effects of removing external Na^+ ions on membrane potential and $[Ca^{2+}]_i$ are summarized in Fig. 4. In the continued presence of either glucose or tolbutamide, replacement of 140 mM Na^+ with 140 mM NMDG, caused membrane repolarization which terminated the Ca^{2+} spike potentials and attenuated the secretagogue-induced rise in $[Ca^{2+}]_i$. Our findings suggest that external Na^+ ions play a fundamental role in regulating the membrane potential of insulin-secreting cells, possibly by providing the net inward current required to maintain a depolarized potential, following K^+ channel closure. These data fit with the findings that (*i*) glucose-stimulated insulin release is reduced in media of lowered extracellular Na^+ (Hales & Milner, 1968*a*; Lambert et al., 1974), (*ii*) under conditions favoring sodium entry insulin secretion is enhanced (Hales & Milner, 1968*b*; Lowe et al., 1976) and (*iii*) that in media deprived of Na^+, glucose loses its ability to inhibit ^{86}Rb efflux (Lebrun, Plasman & Herchuelz, 1989). However, the definitive role of sodium in β-cell stimulus-secretion coupling remains controversial. In 1982 Ribalet and Eddlestone showed that

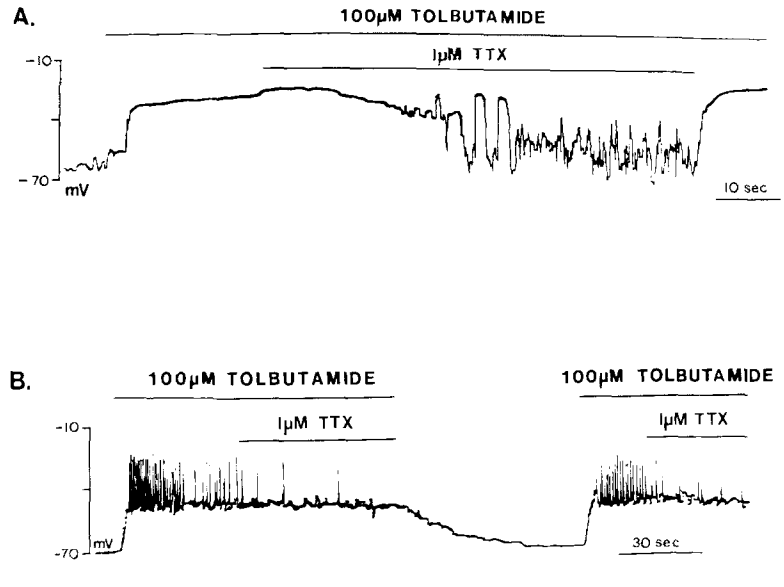


Fig. 6. The action of tetrodotoxin (TTX) (1 μM) upon tolbutamide-induced electrical activity in individual RINm5F cells. The experiment shown in (*A*) began 850 sec after formation of the whole-cell recording configuration and 780 sec after Ca^{2+} spike potentials had run down. The record shows that TTX repolarized the membrane, but the effect upon the potential was complex. The experiment shown in (*B*), began 220 sec after forming the whole cell, illustrating that TTX abolished tolbutamide-induced Ca^{2+} spike potentials

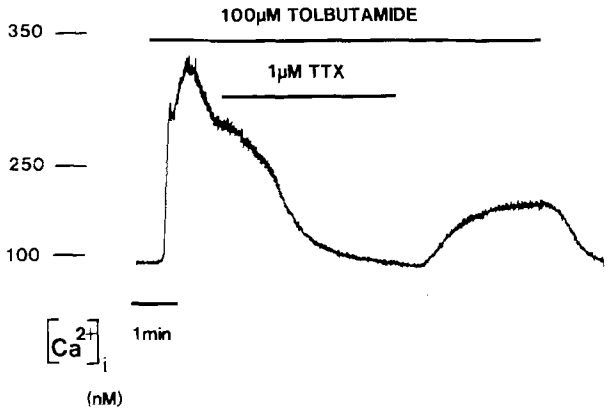


Fig. 7. The effect of tetrodotoxin (TTX) (1 μM) upon the tolbutamide-induced increase in $[Ca^{2+}]_i$ in an individual RINm5F cell

the electrical activity of mouse β-cells was significantly altered by removal of external Na^+. In agreement with our data the membrane repolarized, but this repolarization was only found to be transient and both the rate of rise and peak potential of the Ca^{2+} spike were unaffected. In order to identify the mechanism of Na^+ entry the effects of the specific Na^+ channel blocker tetrodotoxin (TTX) (Kao, 1966) have been investigated. Dean and Matthews (1970) and Meissner and Schmeltz (1974) both report that TTX had no effect on the electrical activity of stimulated β-cells, whereas Meissner and Preissler (1980) and Tarvin and Pace (1981) have shown that TTX in fact depolarizes the cell, while veratridine, a Na-channel agonist, hyperpolarizes the membrane. Conversely, the first report of functional Na-channels in insulin-secreting cells by Donatsch et al. (1977), demonstrated that veratridine caused a sustained release of insulin from rat β-cells and that these effects were reversed by TTX. Tetrodotoxin has also been shown to partially inhibit glucose-induced insulin release, whereas removing extracellular Na^+ produced a more marked inhibition of insulin secretion (Lambert et al., 1974; Donatsch et al., 1977; Hiriart & Matteson, 1988). Two recent patch-clamp investigations have also presented conflicting reports as to the physiological significance of Na^+ channels in these cells. Plant (1988), in mouse β-cells, found that TTX-sensitive voltage-dependent Na^+ channels were completely inactivated at potentials more positive than −80 mV, indicating that they were unlikely to have a major role to play in stimulus-secretion coupling. Hiriart and Matteson (1988), on the other hand, found that TTX-sensitive Na^+ channels were inactivated at potentials around −40 mV.

In our experiments the average RINm5F cell resting membrane potential was found to be approximately −60 mV ($n = 83$). A mean glucose- or tolbutamide-evoked depolarization of about 23 and 27 mV, respectively (Table 1), would place the stimulated membrane potential at about −37 and −33 mV, respectively, i.e., very close to the inactivation potential for Na-channels as predicted by Hiriart and Matteson (1988). The implications of this are that in a number of our experiments the threshold for inactivation would have been exceeded and TTX could not have any effect on the membrane potential. However, since TTX did influence a large number of cells, 88% in all, repolarizing the membrane (Figs. 5 and 6*A*), terminating the generation of Ca^{2+} spike potentials (Figs. 5 and 6*B*) and lowering $[Ca^{2+}]_i$ (Fig. 7), and since voltage-gated TTX-

sensitive Na^+ currents have been described in these cells (Rosman et al., 1986), functional Na^+ channels must play a role in stimulus-secretion coupling.

There is evidence from our experiments that not all the inward Na^+ current is carried by Na^+ channels. First, the magnitude of repolarization brought about by TTX, although sufficient to terminate Ca^{2+}-spikes, is much smaller (on average less than 10 mV) than that seen by removal of external Na^+ (*compare* Figs. 4 and 5). Secondly, in the few experiments where TTX did produce a more marked repolarization, the membrane potential was not stable, as seen in Fig. 6*A*. In this particular experiment tolbutamide was used to close K_{ATP} channels, thereby depolarizing the membrane. When TTX was added in the continued presence of tolbutamide, overall the membrane repolarized, but there were clear oscillations in the potential, suggesting an additional TTX-insensitive component to the inward current.

In normal insulin-secreting cells it has been suggested that external Na^+ ions are not directly involved in regulating the secretion of insulin, but rather play a more passive role by controlling the internal pH (pH_i) through the Na^+/H^+ exchanger (Biden, Janjic & Wollheim, 1986). The effects of secretagogues upon pH_i has not been addressed in this particular study, but Na^+ entry through the Na^+/H^+ exchanger seems unlikely to provide the net inward current, since amiloride had no effect upon either the membrane potential or $[Ca^{2+}]_i$. Furthermore, Na^+ influx through the Na^+/Ca^{2+} exchanger is also unlikely, since the removal of external Na^+ had no effect upon $[Ca^{2+}]_i$ (Fig. 4).

In conclusion, our experiments indicate that following closure of ATP-sensitive K^+ channels (either indirectly, by the metabolic breakdown of glucose or D-glyceraldehyde, or directly through the use of tolbutamide), maintaining the ensuing depolarization of the membrane requires extracellular Na^+. There is evidence that at least part of this current is carried by Na^+ through TTX-sensitive channels.

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