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Cytoplasmic calcium transients due to single action potentials and voltage-clamp depolarizations in mouse pancreatic B-cells

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Changes in the cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) in pancreatic B-cells play an important role in the regulation of insulin secretion. We have recorded $[Ca^{2+}]_i$ transients evoked by single action potentials and voltage-clamp Ca^{2+} currents in isolated B-cells by the combination of dual wavelength emission spectrofluorimetry and the patch-clamp technique. A 500–1000 ms depolarization of the B-cell from −70 to −10 mV evoked a transient rise in $[Ca^{2+}]_i$ from a resting value of ~100 nM to a peak concentration of 550 nM. Similar $[Ca^{2+}]_i$ changes were associated with individual action potentials. The depolarization-induced $[Ca^{2+}]_i$ transients were abolished by application of nifedipine, a blocker of L-type Ca^{2+} channels, indicating their dependence on influx of extracellular Ca^{2+}. Following the voltage-clamp step, $[Ca^{2+}]_i$ decayed with a time constant of ~2.5 s and summation of $[Ca^{2+}]_i$ occurred whenever depolarizations were applied with an interval of <2 s. The importance of the Na^+-Ca^{2+} exchange for B-cell $[Ca^{2+}]_i$ maintenance was evidenced by the demonstration that basal $[Ca^{2+}]_i$ rose to 200 nM and the magnitude of the depolarization-evoked $[Ca^{2+}]_i$ transients was markedly increased after omission of extracellular Na^+. However, the rate by which $[Ca^{2+}]_i$ returned to basal was not affected, suggesting the existence of additional $[Ca^{2+}]_i$ buffering processes.

Key words: B-cell/cytoplasmic Ca^{2+}/Ca^{2+} channels/insulin secretion/Na^+-Ca^{2+} exchange

Introduction

Microelectrode recordings from intact pancreatic islets have shown that glucose stimulation of insulin secretion is associated with the appearance of a characteristic pattern of electrical activity which, at intermediate glucose concentrations (6–15 mM), consists of bursts of Ca^{2+}-dependent action potentials separated by repolarized intervals (Henquin and Meissner, 1984; Ashcroft and Rorsman, 1989). Using the patch-clamp technique many of the ion channels that contribute to the B-cell electrical activity have been characterized (reviews: Petersen and Findlay, 1987; Ashcroft and Rorsman, 1989; Dunne and Petersen, 1991). For example, the Ca^{2+} channels which underlie the B-cell action potentials have been investigated both at the whole-cell (Rorsman and Trube, 1986; Plant, 1988; Satin and Cook, 1988) and single-channel level (Rorsman *et al.*, 1988; Velasco *et al.*, 1988; Smith *et al.*, 1989). Most available evidence argues that in mouse B-cell there is only one type of Ca^{2+} channel (Plant, 1988; Rorsman *et al.*, 1988; buf cf. Hopkins *et al.*, 1991) with properties reminiscent of L-type Ca^{2+} channels in neurons (Nowycky *et al.*, 1985), whereas in rat B-cells there is strong evidence for both T- and L-type Ca^{2+} channels (Ashcroft *et al.*, 1990; Sala and Matteson, 1990). By the combination of microelectrode recordings of membrane potential and fluorimetric measurements of $[Ca^{2+}]_i$, it has recently been possible to demonstrate the correlation between electrical

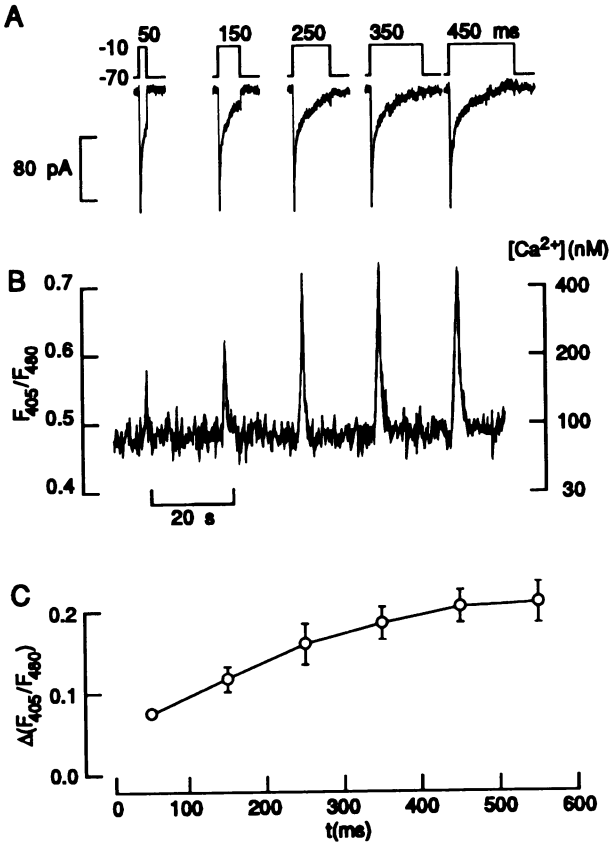

Fig. 1. Relationship between length of depolarization and amplitude of $[Ca^{2+}]_i$ transient. (**A**) Ca^{2+} currents recorded at 2.6 mM extracellular Ca^{2+}. Depolarizations went from a holding potential of −70 to −10 mV, as indicated by the step deflection above each current trace. The duration of the depolarizations was 50–450 ms. The electrode was filled with the Cs^+-containing intracellular solution and TEA (10 mM) was included in the extracellular solution to reduce outward currents due to Cs^+ flux through voltage-dependent K^+ channels. (**B**) Ca^{2+} transients resulting from Ca^{2+} currents shown in (A). The Ca^{2+} currents are positioned above the respective $[Ca^{2+}]_i$ transients. Note the use of different time axes in (A) and (B). (**C**) Peak increases in $[Ca^{2+}]_i$ (F_{405}/F_{480} ratio change) values plotted against duration of depolarization. Mean values ± SEM of four (550 ms) or six (other points) experiments. The F_{405}/F_{480} ratio was determined at 20 Hz.

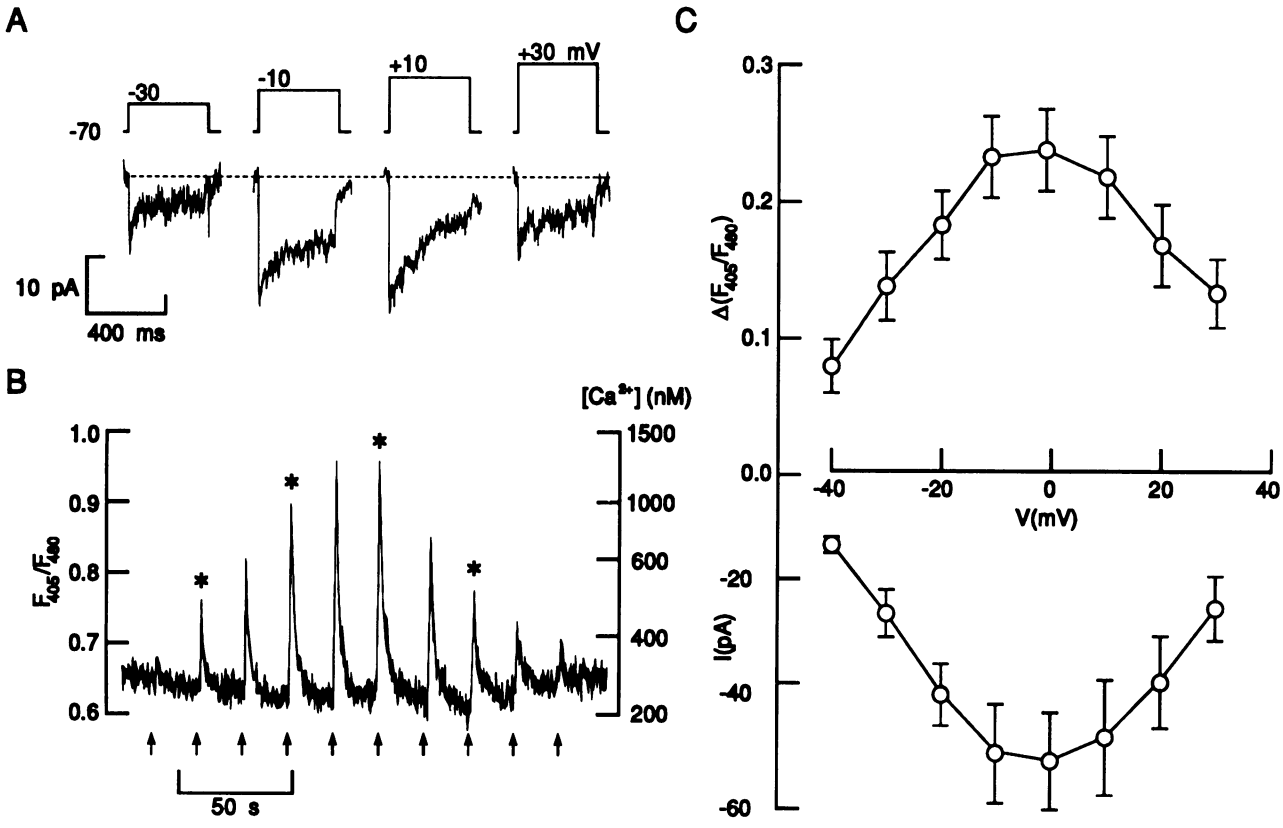

Fig. 2. Voltage dependence of Ca^{2+} currents and associated $[Ca^{2+}]_i$ transients. (**A**) Whole-cell Ca^{2+} currents being observed during 400 ms depolarizations to −30 mV, −10 mV, +10 mV and +30 mV from a holding potential of −70 mV. (**B**) Changes in $[Ca^{2+}]_i$ by Ca^{2+} currents evoked by depolarizations to membrane potentials between −40 and +50 mV. Transients due to currents displayed in (A) are marked by asterisks. Note that different time axes were used in (A) and (B). (**C**) Peak Ca^{2+} currents observed during depolarizing commands (lower curve) and associated changes in $[Ca^{2+}]_i$ (upper curve). Note similar voltage dependence with maximum amplitudes observed at ~0 mV. The F_{405}/F_{480} ratio was determined at 20 Hz.

activity and $[Ca^{2+}]_i$ in intact pancreatic islets (Santos *et al.*, 1991).

Radioisotopic measurements using ^{45}Ca have indicated the importance of the Na^+-Ca^{2+} exchange for $[Ca^{2+}]_i$ maintenance in the B-cell (Wollheim and Sharp, 1981). As in other excitable tissues, the B-cell Na^+-Ca^{2+} exchange is believed to be electrogenic, coupling the outward transport of one Ca^{2+} to the inward movement of three Na^+ (Plasman *et al.*, 1990), but detailed knowledge about its mode of operation is still missing.

Understanding of fundamental cellular processes requires recordings of $[Ca^{2+}]_i$ from voltage-clamped B-cells. The Na^+-Ca^{2+} exchange is an example of a process which is best studied under such experimental conditions since omission of extracellular Na^+ has dramatic effects on the B-cell membrane potential (Ribalet and Beigelman, 1982; de Miguel *et al.*, 1988). In the present study we have combined the patch-clamp technique and microfluorimetric recordings to: (i) delineate the relationship between voltage-clamp Ca^{2+} currents and associated changes in $[Ca^{2+}]_i$; (ii) study the $[Ca^{2+}]_i$ dependence of Ca^{2+} current inactivation (cf. Plant, 1988); and (iii) assess the importance of the Na^+-Ca^{2+} exchange for buffering $[Ca^{2+}]_i$.

Results

Dependence of $[Ca^{2+}]_i$ transients on duration of depolarization

The resting $[Ca^{2+}]_i$ in individual B-cells during standard whole-cell conditions was 110 ± 20 nM (n = 21). Voltage-clamp depolarizations from −70 mV to −10 mV evoked inward Ca^{2+} currents (Figure 1A) and caused transient increases in $[Ca^{2+}]_i$ reaching a maximum peak concentration of 500 nM (Figure 1B). The amplitude of the $[Ca^{2+}]_i$ transients increased with the duration of the depolarization. During the depolarizations the Ca^{2+} currents inactivated and no inward currents were observable at the end of long voltage steps. As a result of the inactivation the relationship between pulse duration and change in $[Ca^{2+}]_i$ was hyperbolic with half-maximal increase being observed following a depolarization of ~150 ms with a maximum amplitude being attained after 400 ms (Figure 1C). $[Ca^{2+}]_i$ rapidly returned to basal at the end of the depolarizations. The decay followed an exponential time course with a time constant of 1.4 ± 0.1 s (n = 5).

Voltage dependence of $[Ca^{2+}]_i$ transients

The dependence of the Ca^{2+} currents and $[Ca^{2+}]_i$ transients on the voltage during depolarizations of 400 ms duration is shown in Figure 2. Depolarizations from a holding potential of −70 mV to voltages between −40 and +50 evoked inward Ca^{2+} current which reached a maximum amplitude between 0 and +10 mV (panel A). The resulting $[Ca^{2+}]_i$ transients are shown in Figure 2B. In being first observable at about −40 mV, increasing up to +10 mV and declining at more positive voltages, the $[Ca^{2+}]_i$ transients have about the same voltage dependence as the underlying Ca^{2+} current. During depolarizations to −10 mV, $[Ca^{2+}]_i$ rose on average by 380 ± 120 nM (n = 5; $P < 0.025$). The

voltage dependence of the amplitude of the $[Ca^{2+}]_i$ transients and Ca^{2+} currents in six different cells is shown in Figure 2C.

$[Ca^{2+}]_i$ transients correlated with action potentials

Pancreatic B-cells are known to generate Ca^{2+}-dependent action potentials (review: Henquin and Meissner, 1984). An attempt was therefore made to observe the $[Ca^{2+}]_i$ transients which should result from Ca^{2+} channel activation during action potentials. Figure 3A shows the electrical activity and associated change in intracellular $[Ca^{2+}]_i$ when releasing the voltage-clamp (holding current: −6.2 pA) in a B-cell stimulated with 10 mM glucose. The action potentials are associated with an increase in $[Ca^{2+}]_i$ from 100 to 400 nM, but it was not possible to correlate a change in $[Ca^{2+}]_i$ to any particular action potential; probably because the frequency was too high to resolve the individual $[Ca^{2+}]_i$ transients.

To improve the resolution we recorded electrical activity from a B-cell in which the action potential frequency had been reduced by injection of a hyperpolarizing current (Figure 3B). Under these experimental conditions it was possible to observe the $[Ca^{2+}]_i$ transients resulting from individual action potentials.

Even larger $[Ca^{2+}]_i$ transients due to single action potentials were seen in the presence of 20 mM TEA (Figure 3C). The long duration of these action potentials is due to blockage of delayed rectifying K^+ channels that normally underlie action potential repolarization in B-cells (Smith *et al.*, 1990). The B-cell was initially held at −70 mV (holding current: −4 pA). Release of the voltage-clamp resulted in spontaneous electrical activity consisting of long action potentials and large $[Ca^{2+}]_i$ transients (>1 μM). It is worthy of note, however, that even with $[Ca^{2+}]_i$ transients as large as this, buffering of Ca^{2+} was completed within a few seconds.

Voltage-gated Ca^{2+} transients in intact B-cells

In the remainder of this study $[Ca^{2+}]_i$ transients were recorded from intact B-cell using the perforated-patch whole-cell technique (Horn and Marty, 1988). The resting $[Ca^{2+}]_i$ averaged 100 ± 10 nM in 35 cells. Figure 4A shows the Ca^{2+} current evoked during a 950 ms depolarization to −10 mV from a holding potential of −70 mV (top), the integrated current (continuous line, below) and the associated change in indo-1 F_{405}/F_{480} fluorescence ratio (circles, below). The Ca^{2+} current inactivated by 90% during the voltage-clamp pulse. It can be noted that Ca^{2+} current inactivation was almost complete before there was any detectable increase in $[Ca^{2+}]_i$. The time course of the integrated current closely follows that of the increase in $[Ca^{2+}]_i$. In 31 different B-cells, a 950 ms depolarization from −70 to −10 mV produced an increase corresponding to 420 ± 60 nM ($P < 0.001$). This is considerably smaller than expected from integration of the Ca^{2+} current. In the cell shown in Figure 4A, which can be regarded as typical, the integrated current amounted to 28×10^{-12} A. This corresponds to 1.5×10^{-16} mol of Ca^{2+}. In a cell with a diameter of 13 μm (cf. Rorsman and Trube, 1986), such an influx of Ca^{2+} is sufficient to elevate $[Ca^{2+}]_i$ to >100 μM assuming no intracellular buffering.

Addition of the dihydropyridine Ca^{2+} channel antagonist nifedipine (20 μM) almost abolished the Ca^{2+} current (not shown but see Rorsman and Trube, 1986) and the resulting

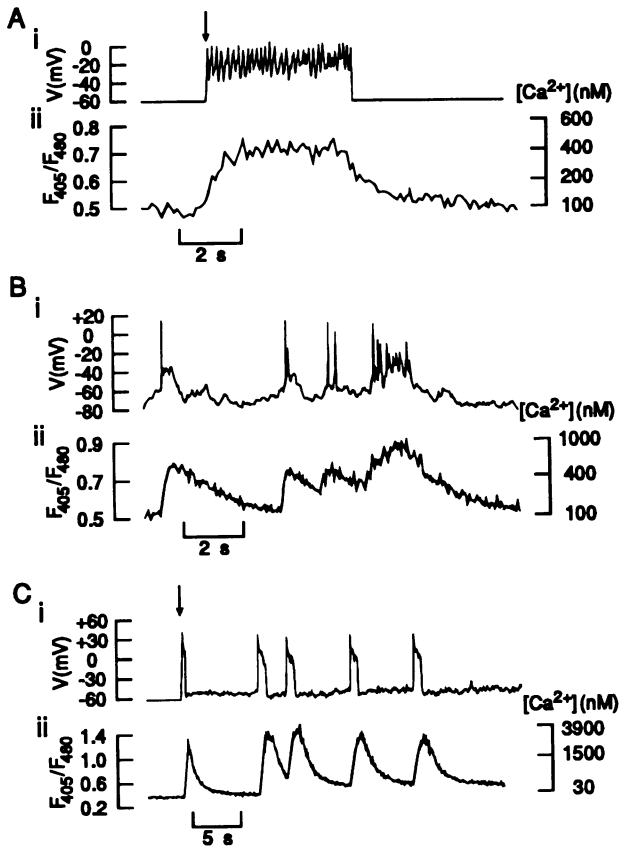

Fig. 3. $[Ca^{2+}]_i$ transients associated with action potentials. (**A**) (top): electrical activity observed when releasing voltage-clamp. Cell was initially held at −60 mV and clamp released as indicated by vertical arrow. Note high frequency of action potentials. (Bottom): associated increase in $[Ca^{2+}]_i$. (**B**) (top): electrical activity observed after increasing extracellular Ca^{2+} concentration to 10.2 mM and reducing action potential frequency by injection of hyperpolarizing current (−5 pA); (bottom): $[Ca^{2+}]_i$ transients evoked by action potentials. Note that when action potentials occur at a high frequency, $[Ca^{2+}]_i$ transients summate. (**C**) (top): electrical activity observed in the presence of 10.2 mM Ca^{2+} and 20 mM TEA^+. Note long duration of action potentials. Cell was initially held at −70 mV and clamp released as indicated by arrow. (Bottom): $[Ca^{2+}]_i$ transients resulting from action potentials. In (A)−(C), the B-cells were stimulated by 10 mM glucose. The F_{405}/F_{480} ratio was determined at 20 Hz.

$[Ca^{2+}]_i$ transient (Figure 4C). Figure 4B illustrates that the decay of $[Ca^{2+}]_i$ at the end of the depolarization could be approximated by a single exponential. In nine different cells the time constant of the decay averaged 2.4 ± 0.4 s.

Summation of $[Ca^{2+}]_i$ transients

Figure 5A shows Ca^{2+} currents and associated changes in $[Ca^{2+}]_i$ evoked by 200 ms depolarizations going to −10 mV from a holding potential of −70 mV applied at a frequency of 0.5 Hz. This voltage range and duration approximate those of the B-cell action potential (Atwater *et al.*, 1979). It is clear that each depolarization evoked a discrete increase in $[Ca^{2+}]_i$. Between the depolarizations, $[Ca^{2+}]_i$ almost returned to basal and little summation of $[Ca^{2+}]_i$ occurred.

When the depolarizations were applied at 1 Hz, the interval was too short for $[Ca^{2+}]_i$ to recover fully but the individual transients still remained distinguishable. By contrast, when depolarizations were applied at 2 Hz, $[Ca^{2+}]_i$ rapidly rose to a plateau and the $[Ca^{2+}]_i$ transients due to the voltage-

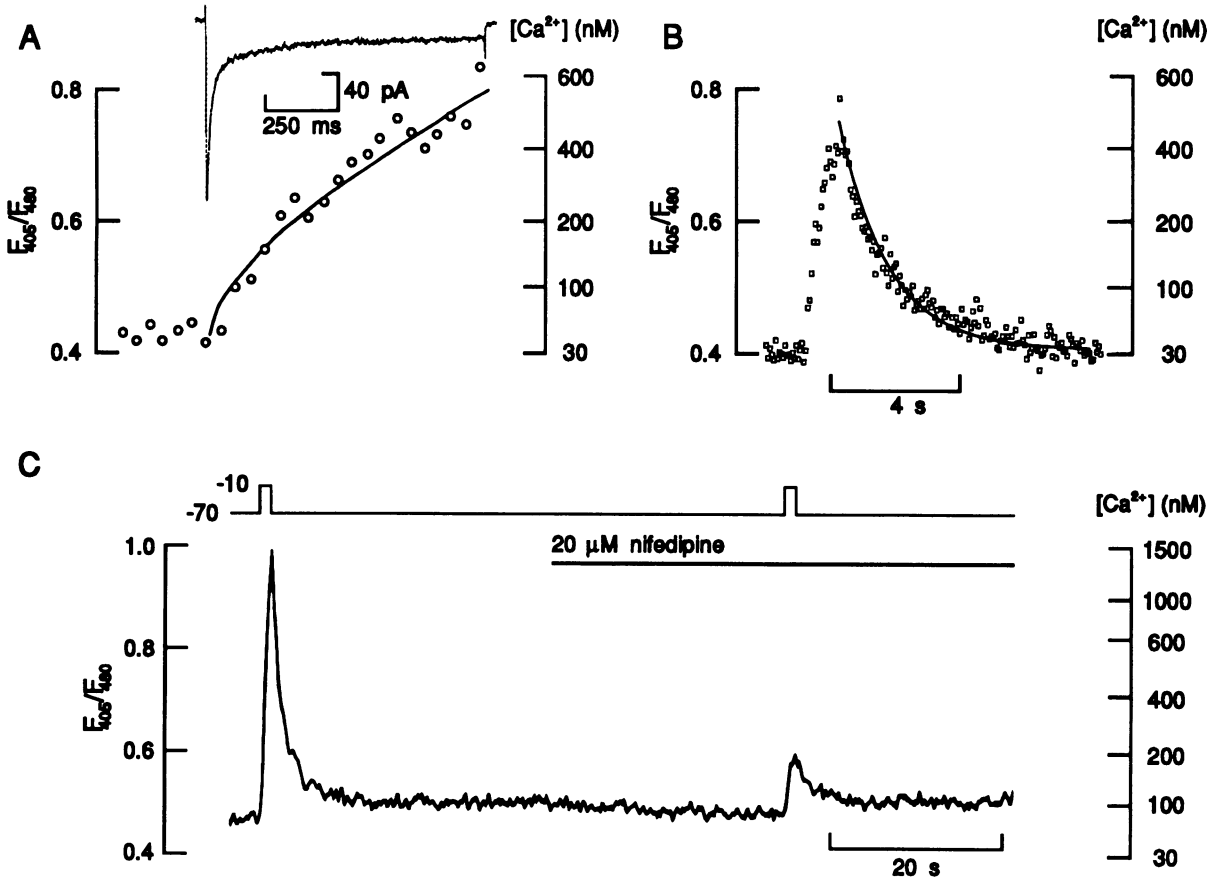

Fig. 4. Time course of the development and recovery of $[Ca^{2+}]_i$ transients. (**A**) (top): Ca^{2+} current recorded from single B-cell during a 950 ms depolarization from −70 mV to −10 mV; (bottom): integrated Ca^{2+} current (continuous line) and observed F_{405}/F_{480} ratio data points (open circles). Note close correlation between integrated current and increase in $[Ca^{2+}]_i$. (**B**) Exponential decay of $[Ca^{2+}]_i$ transient. Squares show the observed F_{405}/F_{480} ratio data points and continuous curve shows exponential function (time constant: 1.4 s) approximated to the data points. (**C**) Block by 20 μM nifedipine. The B-cell was depolarized for 950 ms as shown by the staircase above the fluorescence trace. Nifedipine was added as indicated by horizontal bar. The F_{405}/F_{480} ratio was determined at 10 (C) or 20 Hz (A and B).

clamp depolarizations merged. The observation that $[Ca^{2+}]_i$ rose to a plateau, from which it did not increase further, when the pulse frequency exceeded 1 Hz can be explained by the data summarized in Figure 5D. Whereas the Ca^{2+} current amplitude was almost unchanged when depolarizations are applied at 0.5 Hz (open circles) and 1 Hz (open squares), the amplitude decreased to ~40% of its intial amplitude when depolarizations were applied at 2 Hz (filled circles). Consequently, the influx of Ca^{2+} during high frequency stimulation is just sufficient to balance the buffering of $[Ca^{2+}]_i$ taking place between the depolarizations. Comparison of the data in Figure 5C−D reveals that the time course of the decrease in Ca^{2+} current amplitude when depolarizations were applied at 2 Hz is roughly superimposable on that of the increase in $[Ca^{2+}]_i$ observed under these conditions.

Influence of $Na^+ - Ca^{2+}$ antiport on $[Ca^{2+}]_i$

Figure 6A shows the changes in $[Ca^{2+}]_i$ occurring in response to 950 ms depolarizations from −70 to −10 mV (pulses applied as indicated by arrows) in the presence and absence of extracellular Na^+. Removal of Na^+ produced both an elevation of the resting $[Ca^{2+}]_i$ (+90 ± 40 nM, $n = 11$; $P < 0.05$) and an increased amplitude of the depolarization-evoked $[Ca^{2+}]_i$ transients. In nine different cells, the 950 ms depolarization produced a rise of 460 ± 150 nM under basal conditions. After removal of extracellular Na^+, the amplitude of the depolarization-induced $[Ca^{2+}]_i$ transients increased to 740 ± 130 nM ($P < 0.005$ when compared with the increase under control conditions). However, as illustrated in Figure 6B, the rate of the decline was little affected by Na^+ removal and in four different cells the time constants for the decay averaged 2.2 ± 0.5 s and 2.1 ± 0.2 s ($n = 4$) in the presence (a) and absence (b) of extracellular Na^+, respectively. The increase in the amplitude of the Ca^{2+} transients in the absence of Na^+ cannot be attributed to an increased Ca^{2+} current (Figure 6C), as the Ca^{2+} current amplitude was largely the same in the absence (b) and presence (a and c) of extracellular Na^+. The increase in resting $[Ca^{2+}]_i$ observed after removal of extracellular Na^+ was not dependent on stimulation of Ca^{2+} influx and was observed also when the B-cell was voltage-clamped at −70 mV and no depolarizations applied (not shown).

The larger voltage-evoked $[Ca^{2+}]_i$ transients seen after inhibition of the $Na^+ - Ca^{2+}$ exchange do not reflect Ca^{2+} entry by a route different from the voltage-gated Ca^{2+} channels. This is demonstrated in Figure 7 in which we have utilized nifedipine to block the component due to Ca^{2+} entry through L-type Ca^{2+} channels. It is clear that the

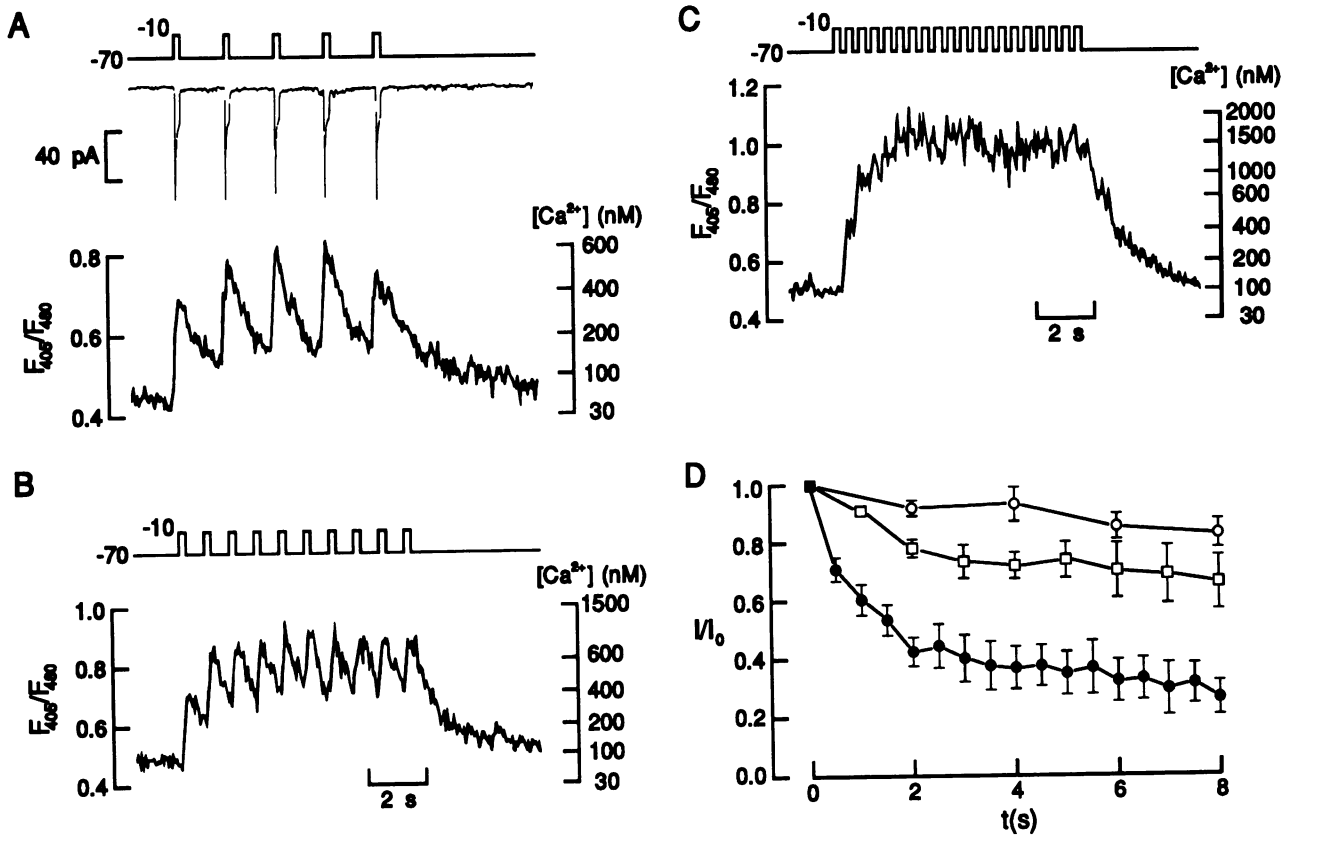

Fig. 5. Summation of $[Ca^{2+}]_i$ transients. (**A**) (top): Ca^{2+} currents evoked by depolarizing the B-cell for 200 ms. Pulses were applied at a frequency of 0.5 Hz as indicated. (Bottom): associated changes in $[Ca^{2+}]_i$. Note that $[Ca^{2+}]_i$ almost recovers completely between the depolarizations. (**B**) (top): 200 ms depolarizations from −70 to −10 mV were applied at a frequency of 1 Hz; (bottom): concomitant changes in $[Ca^{2+}]_i$. Note incomplete recovery of $[Ca^{2+}]_i$ between the pulses. (**C**) (top): 200 ms depolarizations from −70 to −10 m applied at 2 Hz; (bottom): associated change in $[Ca^{2+}]_i$. Note that except for the two first pulses, it is not possible to associate an increase in $[Ca^{2+}]_i$ with any particular depolarization. In (A)−(C), the F_{405}/F_{480} ratio was determined at 20 Hz. (**D**) Relative amplitude of peak Ca^{2+} current plotted against time elapsed after first pulse when depolarizations were applied at 0.5 (open circles), 1 (squares) and 2 Hz (filled circles). Values are means ± SEM of four experiments. The peak current amplitude during the first depolarization was taken as unity.

voltage-gated $[Ca^{2+}]_i$ transient observed in the absence of extracellular Na^+ is as sensitive to nifedipine as that observed under control conditions (cf. Figure 4C).

Discussion

The approach utilized in the present study enables us to investigate the correlation between electrophysiological processes and changes in $[Ca^{2+}]_i$ in individual B-cells. Such recordings are essential for the characterization of the molecular processes involved in the regulation of the B-cell stimulus−secretion coupling.

Mechanisms of Ca^{2+} current inactivation

The B-cell Ca^{2+} current has been reported to be subject to Ca^{2+}-induced (Plant, 1988) and voltage-dependent inactivation (Satin and Cook, 1989). The inactivation/reactivation properties of the Ca^{2+} current have also been proposed to account for the oscillatory electrical activity which characterizes the B-cell at intermediate glucose concentrations (Cook *et al.*, 1991). The experimental protocol used in the present study is well suited for exploring the $[Ca^{2+}]_i$ dependence of Ca^{2+} current inactivation. The finding that the time course of the decrease in Ca^{2+} current amplitude is roughly superimposable on that of the increase in $[Ca^{2+}]_i$, seen when short pulses are applied at high frequency (compare panels C and D of Figure 5), supports the idea that Ca^{2+} current inactivation is a Ca^{2+}-dependent process (Plant, 1988). Surprisingly, inactivation of the Ca^{2+} current during a long voltage-clamp step was more rapid and almost complete before an effect on $[Ca^{2+}]_i$ was detectable (see Figure 4A). However, it should be noted that it is the $[Ca^{2+}]_i$ in the close vicinity of the Ca^{2+} channel which is important for Ca^{2+} channel inactivation (Chad and Eckert, 1984). It seems probable that this $[Ca^{2+}]_i$ rises more rapidly than suggested by the total indo-1 fluorescence signal, which represents an average of the entire cytosol. (cf. Augustine and Neher, 1992).

Mechanisms of $[Ca^{2+}]_i$ buffering in pancreatic B-cells

Comparison of the amount of Ca^{2+} entering the B-cell during a voltage-clamp pulse and the resulting Ca^{2+} transient shows that most (>99%) of the Ca^{2+} is rapidly buffered. This figure is very similar to what has recently been reported for adrenal chromaffin cells (Neher and Augustine, 1992).

The present study provides direct evidence for the importance of the Na^+-Ca^{2+} exchange for $[Ca^{2+}]_i$

buffering in the pancreatic B-cell. Pharmacological agents inhibiting the Na^+-Ca^{2+} exchange are not available and it is common practice to study the importance of this Ca^{2+} extrusion pathway by removal of extracellular Na^+. By using voltage-clamped B-cells it was possible to exclude the possibility that the effects observed upon omission of Na^+ are secondary to changes in the B-cell membrane potential (cf. Ribalet and Beigelman, 1982; de Miguel *et al.*, 1988). The observation that basal $[Ca^{2+}]_i$ was approximately doubled after removal of extracellular Na^+ also when holding the B-cell at −70 mV, which is too negative for electrical activity, indicates that Na^+-Ca^{2+} exchange contributes to Ca^{2+} buffering even at rest. The Na^+-Ca^{2+} exchange in B-cells is believed to be electrogenic (Plasman *et al.*, 1990), thus giving rise to an inward current at the resting potential. This may be the reason why the B-cell repolarizes when extracellular Na^+ is replaced by choline (Ribalet and Beigelman, 1982). Indeed, an inward Na^+-dependent current was observed in a few experiments (data not shown), but it is still premature to conclude that it reflects the Na^+-Ca^{2+} exchange. Further support for the importance of the Na^+-Ca^{2+} exchange for $[Ca^{2+}]_i$ homeostasis in the B-cell comes from the observation that the $[Ca^{2+}]_i$ transients, which could be evoked by depolarizing voltage-clamp steps, were markedly increased after removal of extracellular Na^+. Similar observations have been made in smooth muscle cells (Aaronson and Benham, 1989). The latter results were interpreted in terms of a reversal of the Na^+-Ca^{2+} exchange, mediating Ca^{2+} influx due to the inverted Na^+ gradients. However, such a mechanism cannot explain our findings in the B-cell. This is indicated by our observation that the depolarization-evoked $[Ca^{2+}]_i$ transient was abolished by addition of nifedipine; influx of Ca^{2+} by reversed operation of the Na^+-Ca^{2+} exchange should be expected to be resistant to this selective L-type Ca^{2+} channel blocker (cf. Aaronson and Benham, 1989). To account for the larger $[Ca^{2+}]_i$ transients observed after Na^+ removal, we suggest that the tonic activity of the Na^+-Ca^{2+} exchange serves as an effective Ca^{2+} buffer. Inhibition of the Na^+-Ca^{2+} exchange will accordingly have similar effects to reduced internal buffering.

Although removal of extracllular Na^+ increased both basal $[Ca^{2+}]_i$ and the amplitude of the voltage-gated Ca^{2+} transients, it had only a marginal effect on the time constant of recovery. This suggests that in the B-cell there exist additional high-capacity Ca^{2+} buffering processes. These

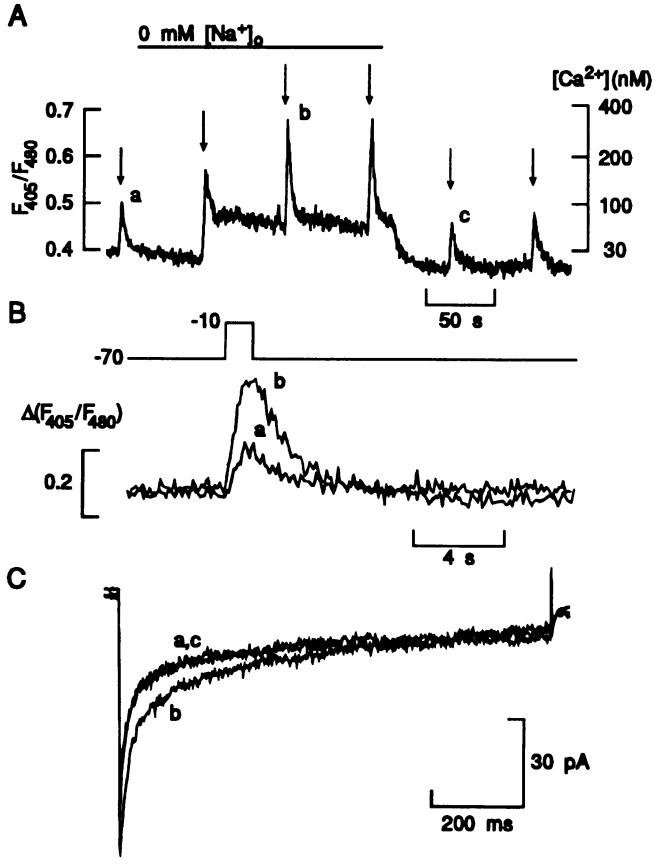

Fig. 6. Influence of Na^+-Ca^{2+} exchange on B-cell $[Ca^{2+}]_i$. (**A**) changes in $[Ca^{2+}]_i$ induced by six depolarizations from −70 to −10 mV (applied as indicated by arrows) and removal of extracellular Na^+ ($[Na^+]_o$). $[Na^+]_o$ was lowered from 140 to 0 mM, by equimolar replacement with $choline^+$, during the period indicated by the horizontal bar. (**B**) $[Ca^{2+}]_i$ transients observed before (**a**) and after (**b**) removal of $[Na^+]_o$. Note increase in magnitude. (**C**) Whole-cell Ca^{2+} currents recorded before (**a**), during (**b**) and after (**c**) the period of $[Na^+]_e$ removal.

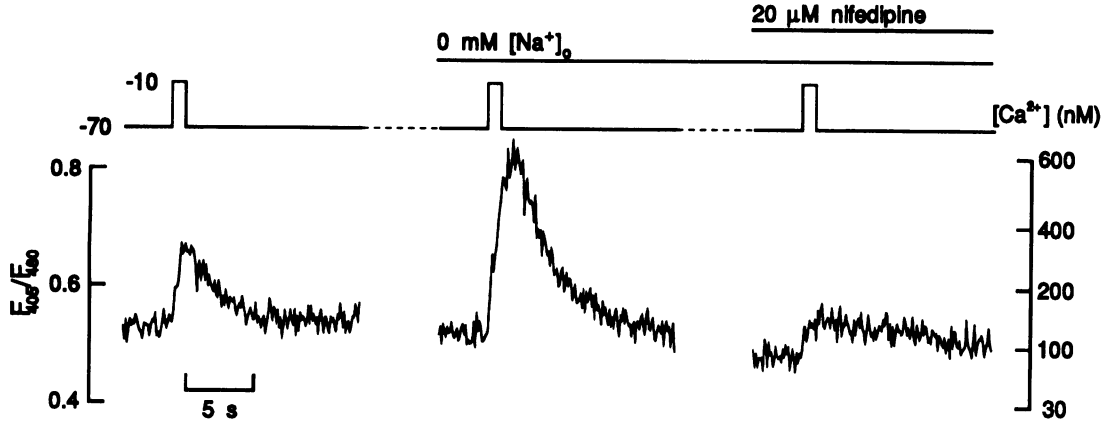

Fig. 7. Effects of Na^+ removal after blockage of Ca^{2+} channels with nifedipine. (**Top**) Depolarizations going from −70 to −10 mV (duration: 950 ms) were applied once every minute. (**Bottom**) Depolarization-induced changes in $[Ca^{2+}]_i$ under control conditions (left), after removal of extracellular Na^+ (replacement with $choline^+$; middle) and after inclusion of nifedipine (20 μM; right) in the perfusion medium. Note increase in amplitude of depolarization-induced $[Ca^{2+}]_i$ transient when Na^+ was removed and inhibition by nifedipine. The F_{405}/F_{480} ratio was determined at 10 Hz.

include the Ca^{2+}-ATPase (Pershadsingh *et al.*, 1981), cytoplasmic Ca^{2+} binding proteins and intracellular organelles. Very recently it was demonstrated that changes in the activity of the plasma membrane Ca^{2+}-ATPase do indeed occur with sufficient speed to account for the rapid buffering of agonist-evoked $[Ca^{2+}]_i$ transients in pancreatic acinar cells (Tepikin *et al.*, 1992). In chromaffin cells, much of the rapid $[Ca^{2+}]_i$ buffering appears to be mediated by a low-affinity immobile Ca^{2+} buffer with properties reminiscent of the calmodulins or annexins (Neher and Augustine, 1992). With regard to Ca^{2+} binding proteins, it is of interest to note that the B-cell contains 15–50 μM calmodulin (Sugden *et al.*, 1979; Valverde *et al.*, 1979; Landt *et al.*, 1982). When allowance is made for the fact that each molecule has four Ca^{2+} binding sites, this corresponds to a buffering capacity of up to 200 μM Ca^{2+}.

$[Ca^{2+}]_i$ transients are longer than the electrophysiological processes

One of the more important observations of this study is that the recovery of $[Ca^{2+}]_i$ following an action potential or a voltage-clamp depolarization is relatively slow and can be described by a single exponential with a time constant of 2 s. This is much longer than the dissociation of Ca^{2+} from indo-1 (dissociation rate constant: 130 s^{-1}; Jackson *et al.*, 1987) and the rate of unbinding from the dye does consequently not limit the temporal resolution. It can thus be concluded that Ca^{2+} is present as a second messenger much longer than suggested by the duration of the underlying electrophysiological processes. The slow rate of $[Ca^{2+}]_i$ buffering also suggests that whenever action potentials are generated at a frequency higher than 1 Hz, the interval is insufficient for recovery of $[Ca^{2+}]_i$ leading to summation of the $[Ca^{2+}]_i$ transients. The significance of these considerations is illustrated by the fact that the B-cell action potential frequency under steady-state conditions is 4 Hz (Atwater *et al.*, 1979). It is therefore not surprising that previous recordings of $[Ca^{2+}]_i$ in single B-cells have failed to detect the $[Ca^{2+}]_i$ transients due to single action potentials (Pralong *et al.*, 1989; Grapengiesser *et al.*, 1991). The present study provides direct evidence that the B-cell action potential, which lasts 50–150 ms (Atwater *et al.*, 1979; Lebrun and Atwater, 1985), is indeed associated with sufficient Ca^{2+} influx to increase $[Ca^{2+}]_i$. It will be interesting to determine if the increase in $[Ca^{2+}]_i$ due to a single action potential is sufficient to intiate exocytosis or whether larger $[Ca^{2+}]_i$ transients, i.e. those resulting from a burst of action potentials, are required. Data from melanotrophs suggest that the relationship between $[Ca^{2+}]_i$ and secretion is not linear and that a certain threshold of $[Ca^{2+}]_i$ must be exceeded before the exocytotic machinery is activated (Thomas *et al.*, 1990). Our preliminary data suggest that this is also the case in the insulin-secreting B-cells.

Materials and methods

Cells

Mouse pancreatic islets were isolated by collagenase digestion and single cells prepared as previously described (Arkhammar *et al.*, 1987). Isolated cells were plated on glass cover-slips (diameter: 22 mm) and cultured in RPMI 1640 tissue culture medium containing 5 mM glucose, 10% (v/v) fetal calf serum and supplemented with 100 μg/ml streptomycin and 100 i.u./ml penicillin. In this preparation, >90% of the cells correspond to B-cells (Nilsson *et al.*, 1987). Cover-slips were sealed over a circular hole (diameter: 20 mm) in a stainless-steel plate, making a recording and perfusion chamber with the cover-slip forming the base. The experimental chamber could be perfused and exchange of the solution was complete within 1 min.

Electrophysiology

Voltage-clamp and membrane potential recordings were made using the standard whole-cell (Hamill *et al.*, 1981) or the perforated-patch whole-cell (Horn and Marty, 1988) configurations. Membrane currents and potentials were measured using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Voltage steps were generated, digitized and stored in a computer (IBM XT-clone) using a Labmaster ADC and the program pClamp (Axon Instruments, Inc., Foster City, CA, USA). Ca^{2+} currents were filtered at 0.3–1 kHz (−3 dB-point: 8-pole Bessel filter) and digitized at 1–2 kHz. Membrane potentials were recorded on video tape using a VCR and a modified digital audio processor (PCM-F1, Sony, Tokyo, Japan) pending analysis.

Fluorescence measurements

$[Ca^{2+}]_i$ was estimated from indo-1 fluorescence by the ratio method using single wavelength excitation and dual wavelength emission (Grynkiewcicz *et al.*, 1985; Benham, 1989). The measurements were performed using the hard- and software of the Newcastle Photometric System (Newcastle, UK) adapted to a Zeiss Axiovert-10 microscope. Excitation was provided at 360 nm by a Zeiss XBO xenon arc lamp and emitted light, after splitting by a dichroic mirror, was detected by two parallel photomultipliers at 405 and 480 nm, respectively. Signal processing was performed on-line using a Compaq 386S computer and the ratio of the fluorescence detected at 405 and 480 nm (F_{405}/F_{480}) was determined at 10 or 20 Hz, as indicated in the figure legends. $[Ca^{2+}]_i$ is given as both the F_{405}/F_{480} ratio and estimated concentrations. Calibration was performed intracellularly using the standard whole-cell configuration and dialysing the cell interior with seven different mixtures of Ca^{2+} and EGTA (Calcium Calibration Buffer Kit II; P/N C-3009, Molecular Probes, Eugene, OR), with free Ca^{2+} concentrations ranging between 16.7 nM and 39.2 μM, supplemented with 0.1 mM indo-1. A new cell was used for each measurement. The ratio was determined in four to five different cells at each concentration of Ca^{2+}. It was assumed that the indicator behaves identically under standard whole-cell and perforated-patch whole-cell conditions. During standard whole-cell recordings, using indo-1-containing electrodes, the background values were measured in the cell-attached configuration before establishing whole-cell conditions. The background amounted to 10–20% of that obtained after equilibration of the indicator. During recordings on intact cells, preloaded with indo-1/AM, background values were negligible.

Solutions

The standard extracellular solution consisted of (in mM) 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES–NaOH (pH 7.4) and 10–20 mM glucose. In some experiments, tetraethylammonium (TEA, 10–20 mM) was included in the extracellular medium. In the membrane potential recordings, the pipette solution was composed of (in mM) 145 KCl, 1 $MgCl_2$, 5 HEPES–KOH (pH 7.15), 3 Mg-ATP and 0.1 indo-1 (potassium salt; Molecular Probes). For the Ca^{2+} current measurements CsCl was equimolarly substituted for KCl. Whenever Na^+ was omitted from the extracellular solution, it was equimolarly replaced by choline-Cl or LiCl and pH adjusted to 7.4 using KOH (addition of 2.5 mM; the initial K^+ concentration being reduced to 3.1 mM). Similar results were obtained with Li^+ and $choline^+$. Electrophysiological recordings from intact B-cells were performed using the perforated-patch whole-cell method (nystatin technique) and a pipette solution consisting of (in mM) 76 Cs_2SO_4, 10 NaCl, 10 KCl, 1 $MgCl_2$, 5 HEPES–KOH (pH 7.35) and 50 μg/ml nystatin (Sigma). It should be emphasized that in the latter experiments, intracellular pH (pH_i) is likely to be clamped to that of the pipette solution since the nystatin pores are very permeable to positive ions with a molecular weight of <200 Daltons (Horn and Marty, 1988). The observed changes in $[Ca^{2+}]_i$ obtained upon omission of extracellular Na^+, a condition known to be associated with acidification of the cytoplasm (Juntti-Berggren *et al.*, 1991), are consequently unlikely to be secondary to changes in pH_i. In the experiments employing nystatin, the cells were preloaded with 1 μM indo-1/AM for 25–35 min prior to the electrophysiological experiments. This resulted in fluorescence intensities similar to those observed when dialyzing the cell with 0.1 mM indo-1. Experiments using the standard whole-cell configuration were carried out at room temperature (20–22°C), whereas experiments on intact B-cells were performed at 30–32°C.

Data analysis

Data are presented as mean values ± SEM. Exponential or hyperbolic functions were fitted to the data points and the time constants calculated using the program Asystant.

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