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PROPERTIES AND CALCIUM-DEPENDENT INACTIVATION OF CALCIUM CURRENTS IN CULTURED MOUSE PANCREATIC B-CELLS

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SUMMARY

1. Ca^{2+} currents were recorded using the whole-cell mode of the patch-clamp technique from mouse pancreatic B-cells kept in culture for 1–4 days. B-cells were identified in the cell-attached mode by their response to a change in the glucose concentration from 3 to 15 or 20 mM or by their inward currents.

2. Only one component of Ca^{2+} current was observed in these cells, which activated at potentials > -50 mV and was blocked by nitrendipine (5 μM), and increased in amplitude by CGP 28392 (5 μM).

3. During maintained depolarizations the Ca^{2+} current inactivated considerably but not completely. Inactivation was most marked at potentials where the Ca^{2+} currents were large, but in general was slower for currents at potentials > 0 mV than at more negative potentials.

4. Two-pulse experiments showed that the inactivation curve for the Ca^{2+} current was U-shaped, returning to unity at potentials approaching the Ca^{2+} equilibrium potential. Measurements of Ca^{2+} entry showed that inactivation was dependent on the amount of Ca^{2+} entering during the pre-pulse, independent of the pre-pulse potential.

5. Ca^{2+} currents were not appreciably slowed when BAPTA, a faster buffer of Ca^{2+}, replaced EGTA in the pipette solution.

6. Replacement of Ca^{2+} in the external solution by Ba^{2+} increased the amplitude of the inward current and largely abolished inactivation. Large inward currents through Ca^{2+} channels were observed in the absence of divalent cations in the external solution (+EGTA), which were presumably carried by Na^{+}. These currents did not inactivate during 150 ms depolarizations, but were increased in amplitude by CGP 28392 (5 μM) and blocked by D600 (30 μM).

7. The observations suggest that normal mouse pancreatic B-cells have only one type of Ca^{2+} channel which is dihydropyridine sensitive and inactivates by a mechanism which is almost purely Ca^{2+} dependent. Inactivation of the Ca^{2+} current will probably be important in the control of Ca^{2+} entry during glucose-induced electrical activity.

INTRODUCTION

Ca^{2+} entry through voltage-dependent Ca^{2+} channels, giving rise to slow-wave and spike activity, is an important step in the sequence of events by which glucose

induces insulin release in pancreatic B-cells. Repolarization of the spikes and slow waves is due to the activation of voltage-dependent, and voltage- and Ca^{2+}-dependent K^+ channels, but inactivation of Ca^{2+} channels may also be involved (for reviews see Henquin & Meissner, 1984; Matthews, 1985; Petersen & Findlay, 1987).

Ca^{2+} currents have been described in neonatal rat B-cells (Satin & Cook, 1985), mouse B-cells (Rorsman & Trube, 1986) and clonal insulin-producing RINm5F cells (Findlay & Dunne, 1985; Rorsman, Arkhammar & Berggren, 1986). In mouse B-cells, Rorsman & Trube (1986) showed that the Ca^{2+} current activated rapidly but inactivated only weakly during long depolarizations. This inactivation was most marked at potentials where the Ca^{2+} current was largest suggesting that Ca^{2+} current inactivation may be Ca^{2+} dependent (for review see Eckert & Chad, 1984).

Inactivation of macroscopic Ca^{2+} currents may have voltage- and Ca^{2+}-dependent components depending on the types of Ca^{2+} channels present. Three main types of Ca^{2+} channels have been described in vertebrates and their distribution varies between tissues (Miller, 1987; Tsien, Hess, McCleskey & Rosenberg, 1987; Fox, Nowycky & Tsien, 1987*b*). In non-neuronal tissues two types of Ca^{2+} channels have been identified: T-type or transient low-voltage-activated channels, characterized by their activation at strong negative membrane potentials and voltage-dependent inactivation, and L-type or high-voltage-activated channels which display Ca^{2+}- and voltage-dependent inactivation in heart cells and neurones. These channels may also be characterized by their pharmacology, having different sensitivities to organic and inorganic Ca^{2+} channel blockers (Carbone & Lux, 1984; Nowycky, Fox & Tsien, 1985; Nilius, Hess, Lansman & Tsien, 1985; Fox, Nowycky & Tsien, 1987*a*, *b*).

In this paper the properties and mechanism of inactivation of Ca^{2+} currents in mouse pancreatic B-cells were investigated in more detail. The results showed that only one population of Ca^{2+} channels is present in the B-cell and that inactivation of the Ca^{2+} currents is largely Ca^{2+} dependent. Some of these results have been presented previously (Plant, 1987*b*, *c*).

METHODS

Preparation

Mouse pancreatic islet cells were isolated and cultured as described previously (Lernmark, 1974; Rorsman & Trube, 1985). Briefly, islets, isolated by collagenase digestion, were dispersed by treatment with a low Ca-EGTA solution, and the single cells and clusters of cells thus obtained kept in culture for 1–4 days in RPMI 1640 culture medium containing 11·1 mM glucose (Gibco, Eggenstein, F.R.G.) supplemented with 10% fetal calf serum. The cells used in the experiments had diameters of between 10 and 15 μm and cell capacitance of 3·3–8·9 pF (mean $5{\cdot}6 \pm 0{\cdot}2$ pF, $n = 53$). B-cells were identified by their response to a change in the glucose concentration from 3 to 15 or 20 mM in the cell-attached mode (Ashcroft, Harrison & Ashcroft, 1984; Rorsman & Trube, 1985; 1986). Many cells did not respond to glucose, particularly on day 4 after their preparation, but had otherwise identical currents to those shown by B-cells. These have been included with the data presented here. Only single cells were used in these experiments.

Recordings

Responses to glucose and Ca^{2+} currents were recorded using the cell-attached and whole-cell mode of the patch-clamp technique respectively (Hamill, Marty, Neher, Sakmann & Sigworth, 1981), with an EPC 7 patch-clamp amplifier (List Electronics, Darmstadt, F.R.G.). Pipettes with

resistances of 4–5 MΩ, when filled with 140 mM-KCl solution, were made of borosilicate glass (Clark Electromedical Instruments, Science Products Trading, Frankfurt, F.R.G.). Pipettes were coated with Sylgard 184 (Dow Corning, Midland, MI, U.S.A.). The patch-clamp amplifier was used to compensate capacitance and series resistance. Experiments were performed in a chamber with a volume of 0·6 ml which was continuously perfused (approximately 1 ml/min) with solutions at room temperature (20–22 °C).

Solutions

The normal bath solution, used in the cell-attached mode, contained (mM): NaCl, 140; KCl, 5·6; $CaCl_2$, 2·6; $MgCl_2$, 1·2; HEPES-NaOH, 10; pH 7·4, and 3, 15 or 20 mM-glucose. To block K^+ currents in whole-cell experiments, the solution was modified to include 20 mM-tetraethylammonium (TEA^+) chloride (replacing Na^+) and KCl was often omitted. Tetrodotoxin (TTX), 1·5 or 3 μM, was added in most experiments to block the Na^+ current present in some B-cells (Plant, 1987*a*, 1988). Pipettes were filled with a solution containing *N*-methyl-D-glucamine chloride (NMG-Cl) to reduce current through K^+ channels. It contained (mM); NMG, 145; HCl, 120; $MgCl_2$, 4; Na_2ATP, 3; $CaCl_2$, 2; EGTA, 10; HEPES, 10; pH 7·15. The pH of the solution was adjusted with NMG. In some experiments, EGTA (ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid) was replaced by 7 mM-BAPTA (1,2-bis(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid). BAPTA was used since it buffers Ca^{2+} approximately five times more rapidly than EGTA (Tsien, 1980; Marty & Neher, 1985). The free $[Ca^{2+}]$ in the solutions was calculated from the stability constants for EGTA (Martell & Smith, 1974) and BAPTA (Tsien, 1980), and was $4·9 \times 10^{-8}$ and $4·0 \times 10^{-8}$ M respectively. The equilibrium buffering capacities,

$$d[Ca^{2+}]_t/d[Ca^{2+}]_f = K'[L]+1,$$

where $[Ca]_t$ is the total concentration of calcium, $[Ca^{2+}]_f$ the free Ca^{2+} concentration, K' the apparent stability constant and [L] the ligand concentration (Marty & Neher, 1985), were $4·1 \times 10^4$ (EGTA) and $4·2 \times 10^4$ (BAPTA). When used the dihydropyridines, nitrendipine (Bayer, Leverkusen, F.R.G.) and CGP 28392 (4-[2-difluoromethoxyphenyl]-1,4,5,7-tetrahydro-2-methyl-5-oxofuropyridine-3-carboxylic acid ethyl ester; Ciba-Geigy AG, Basle, Switzerland), were dissolved in dimethylsulphoxide. Aliquots of the stock solutions (25 mM) were added to the media shortly before use. Nitrendipine stock solutions were kept in the dark, but no special precautions were taken to protect nitrendipine from the light during the experiments.

Data analysis

Currents recorded in the cell-attached mode were digitized (Sony PCM 501/ES) and stored on videotape. In the whole-cell mode, command pulses were generated by computer (DEC 11/23; Hof, 1986) and the resulting membrane currents, filtered at 2 kHz (−3 dB), sampled by computer and stored on floppy discs. Further analysis of the data was performed off-line. The linear leakage current estimated from hyperpolarizing voltage steps between −70 and −120 mV was scaled and subtracted from the currents during depolarizations. Where appropriate results are presented as mean ± standard error of the mean (S.E.M.).

RESULTS

Voltage dependence of Ca^{2+} currents

In many cells it was possible to record Ca^{2+} currents in 2·6 mM-Ca^{2+}. However, in others Ca^{2+} currents were small in spite of a high input resistance. Thus, in most experiments 10·4 mM-Ca^{2+} was preferred to allow measurement of Ca^{2+} currents in the majority of cells. In 10·4 mM-Ca^{2+}, an inward current was observed in all cells on depolarization from a holding potential of −70 mV to potentials less negative than −50 mV (Fig. 1*A*). This current had a similar activation time course and voltage dependence (Fig. 1*D*) to Ca^{2+} currents described previously in mouse B-cells (Rorsman & Trube, 1986). Changing the holding potential in the range between −50

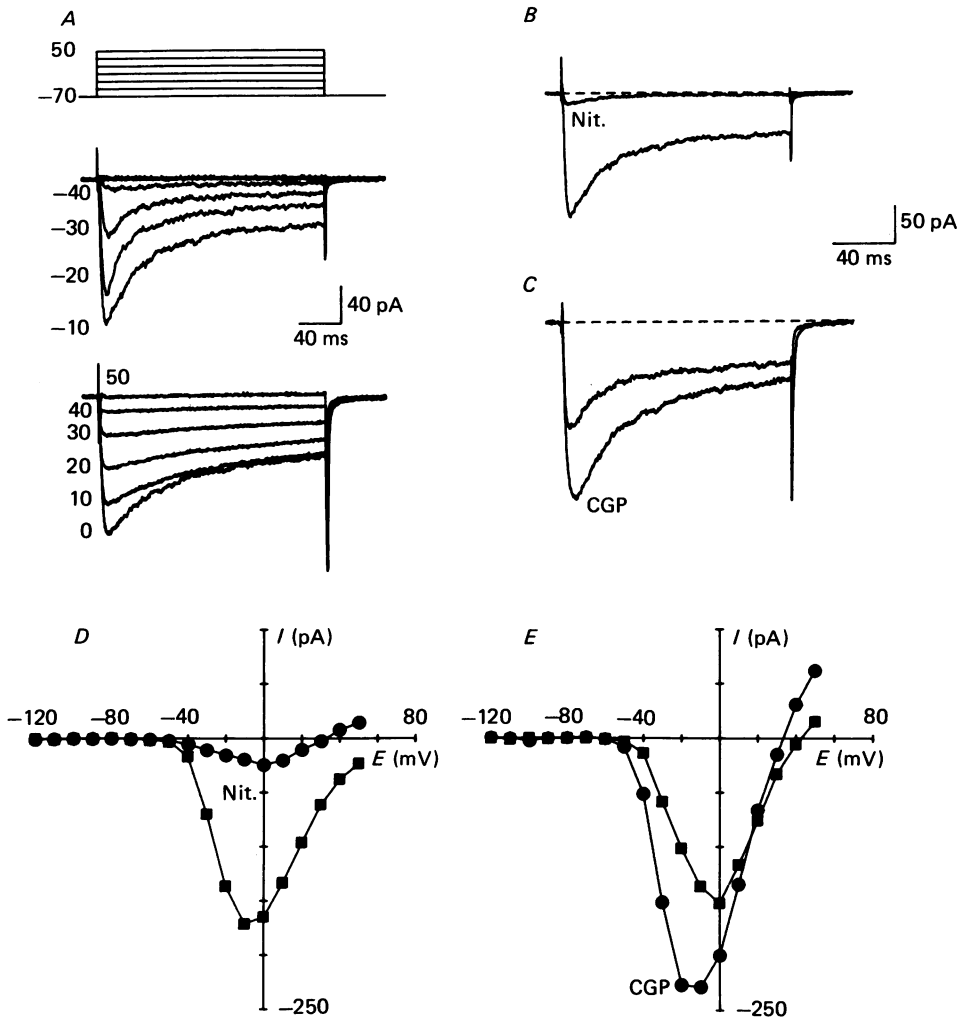

Fig. 1. Ca^{2+} currents and their sensitivity to dihydropyridines. *A*, original current records, not leakage corrected, recorded during 150 ms depolarizations to the indicated potentials (mV) from a holding potential of −70 mV in 10·4 mM-Ca^{2+}. *B*, block of inward currents by 5 μM-nitrendipine (Nit.). *C*, increase in inward currents with CGP 28392 (5 μM). *B* and *C*, potential steps from −70 to −10 mV. *D*, leak-corrected current–voltage relations for currents from the cell shown in *A* and *B* in the control (■) and following application of nitrendipine to the bath solution (●). *E*, current–voltage relations for control (■) and after external application of CGP 28392 (●), same cell as *C*.

and −100 mV had no effect on the amplitude or the time course of the inward currents observed.

The maximum inward current was at potentials between −10 and 0 mV, and in cells with a very low leakage conductance, e.g. the cell in Fig. 1*D*, no reversal of the net leakage-corrected current was observed with test pulse potentials (E) < +50 mV. Larger depolarizations were usually not applied to prevent cell damage. In many

cells, however, the current reversed at potentials between +30 and +50 mV probably owing to rectification of the leakage current. Leakage rectification was observed when ionic currents were blocked, and tended to increase during the course of whole-cell measurements. The amplitude of the Ca^{2+} current, even when normalized to the cell capacitance, and the stability of the recordings were very variable from cell to cell. Some cells became leaky 10 min after dialysis, others were stable for up to 30 min. Similarly, rapid run-down of Ca^{2+} currents was observed in some cells. No clear correlation between run-down and the series resistance (R_s), which would indicate a loss of diffusible factors from the cell into the pipette, was observed.

Ca^{2+} currents were decreased by 5 μM-nitrendipine (Fig. 1*B*). The inward current remaining in nitrendipine had a similar voltage dependence (Fig. 1*D*) and time course to the inward currents in the absence of the Ca^{2+} channel blocker. At −10 mV the time course was similar, and at +10 mV inactivation was more rapid in nitrendipine. This is probably due to an action of the drug (see e.g. Hess, Lansman & Tsien, 1984). There is no evidence to suggest that the current remaining in 5 μM-nitrendipine is through a different type of channel. Rorsman & Trube (1986) also showed that Ca^{2+} currents were blocked by the dihydropyridine nifedipine. On the other hand, Ca^{2+} currents were increased in amplitude by another dihydropyridine CGP 28392, particularly at more negative potentials (Fig. 1*C* and *E*). A larger increase of the Ca^{2+} current at more negative potentials has also been observed in other tissues with Bay K8644, and may be explained by a shift of the voltage dependence of Ca^{2+} current activation to more negative potentials in the presence of the drug (Hess *et al.* 1984; Sanguinetti, Krafte & Kass, 1986). The cross-over of the current–voltage relations at more positive potentials and the change in reversal potential in CGP 28392 probably occur as a result of an increase in non-linear leakage current with time during the experiment. The results with dihydropyridines also indicate that the Ca^{2+} current in B-cells is carried only through L-type channels.

Time course of Ca^{2+} currents

It is clear from Fig. 1*A* that Ca^{2+} currents inactivated with time in this tissue. The rate and extent of inactivation was very variable in different cells. Under the conditions used, the decay is unlikely to be due to the activation of K^+ current. No evidence for time-dependent outward currents was obtained when the pipette contained NMG^+ and the external solution, TEA^+. As the potential was increased the rate of inactivation increased (see records in Fig. 1*A* at −40, −30 and −20), then decreased as the membrane was further depolarized. This is an indication for Ca^{2+}-dependent inactivation, which would predict that more inactivation occurs when Ca^{2+} entry is increased. It would be expected that currents of similar amplitude inactivate to a similar extent independent of the membrane potential. However, this was not the case in the B-cell. In most cells, as in Fig. 1*A*, there was a considerable slowing of the current at potentials > -20 or −10 mV. Currents at −20 mV inactivated more rapidly than those at +10 mV in Fig. 1*A* even though their amplitudes were similar. This would not be expected if inactivation depends simply on the total amount of Ca^{2+} entering the cell. The observation does not however exclude a Ca^{2+}-dependent mechanism (see Discussion). Thus, even though $[Ca^{2+}]_i$

should be buffered to $\sim 5 \times 10^{-8}$ M by 10 mM-EGTA, Ca^{2+} may accumulate sufficiently to produce inactivation. The time course of inactivation was very different in individual cells. In some, inactivation was well fitted by a single exponential but in others two or more exponential functions were necessary. For this reason, no attempt was made to fit and compare the kinetics of the currents from different cells.

Dependence of current time course on $[Ca^{2+}]_o$

If inactivation is dependent on Ca^{2+} entry, changes in $[Ca^{2+}]_o$ should influence the time course of inactivation. Figure 2 shows superimposed records of currents in 1 and

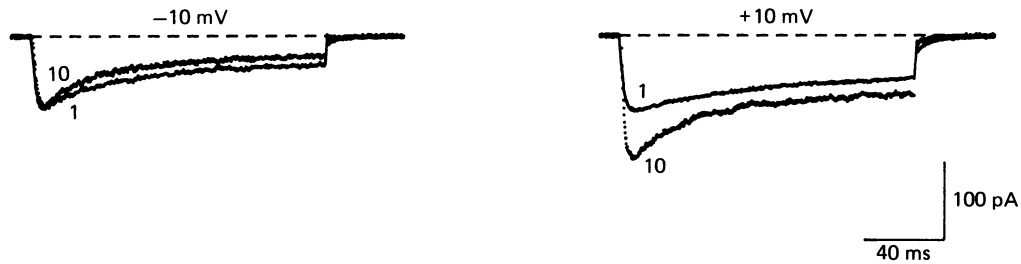

Fig. 2. Effects of external Ca^{2+} on Ca^{2+} currents. Currents from the same cell recorded first in 1 mM then in 10 mM [$Ca^{2+}]_o$ at the potentials indicated.

10 mM-Ca^{2+} at the same membrane potentials. The threshold for activation of the Ca^{2+} current was shifted by $\sim$ 10 mV in 10 mM-Ca^{2+} compared to 1 mM-Ca^{2+}, and the maximum inward current increased and shifted. In the example in Fig. 2, the current at −10 mV was of similar amplitude in 1 and 10 mM-Ca^{2+}, owing to the cross-over of the current–voltage relations. The current in 10 mM-Ca^{2+} inactivated more rapidly and more completely during a 150 ms pulse. At +10 mV the inward current was larger in 10 mM-Ca^{2+} and inactivated more rapidly than in 1 mM-Ca^{2+}. The more rapid inactivation of currents of a similar size would not be predicted from simple models of Ca^{2+}-dependent inactivation (see e.g. Standen & Stanfield, 1982; Chad, Eckert & Ewald, 1984).

Voltage dependence of inactivation

To measure the voltage dependence of inactivation, a test pulse to −10 mV was preceded by a 200 ms pre-pulse to various potentials; test pulse and pre-pulse were separated by an interval of 10 ms to allow most activation to return to its resting state. In Fig. 3*A* the records show the effect of pre-pulses to −30, +10 and +50 mV on the current during the test pulse. The larger current during the test pulse in each record is the control, a test pulse from a holding potential of −70 mV in the absence of a pre-pulse. Controls were applied at regular intervals, two records with a pre-pulse were bracketed by two controls. The records show that the current in the test pulse is reduced to a greater extent when the Ca^{2+} current during the pre-pulse is large. In the presence of a pre-pulse, the test-pulse current kinetics are changed as would be expected if Ca^{2+} entry is reduced. Inactivation was expressed as the ratio of the current during a test pulse after a pre-pulse (I) relative to the current during

a control test pulse (I_c). The values thus obtained are plotted in Fig. 3*B* for two cells which showed different degrees of inactivation. Mean values for four to twelve cells are shown in Fig. 3*C*. Ca^{2+} current during the test pulse was only inactivated at potentials more positive than −50 mV, the potential at which Ca^{2+} current is first activated. Inactivation was greatest around 0 mV, where the Ca^{2+} current is largest,

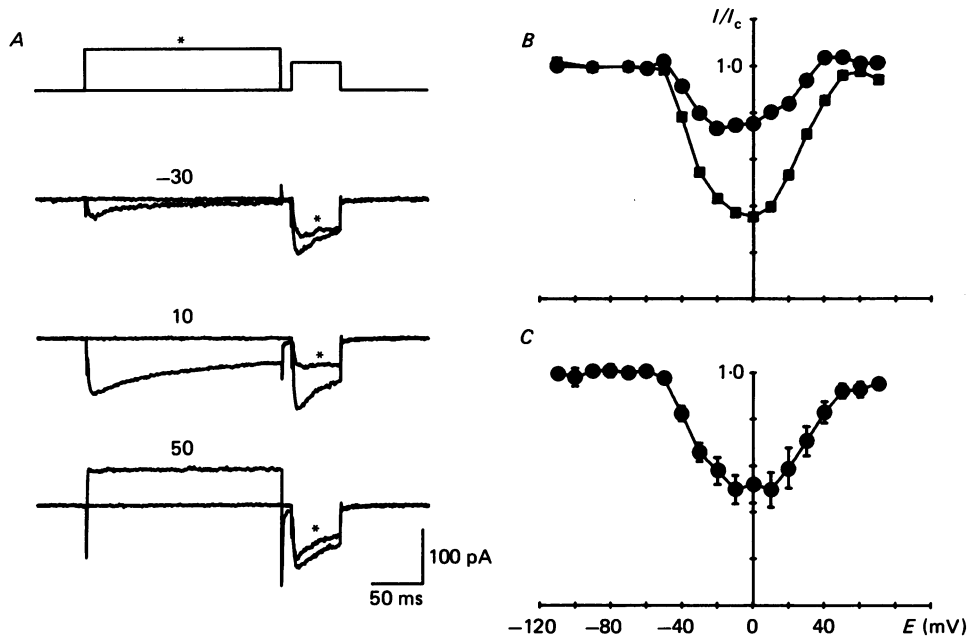

Fig. 3. Inactivation of Ca^{2+} currents. *A*, Ca^{2+} currents recorded during a 50 ms test pulse to −10 mV without, and with (*) a 200 ms conditioning pre-pulse to the potential indicated (mV, records not corrected for leakage). *B*, inactivation curve calculated from records such as those in *A* for two cells which showed different degrees of inactivation. The peak current during the test pulse with a pre-pulse (I) divided by the test-pulse current in the absence of a pre-pulse (I_c) is plotted against the pre-pulse potential. *C* as *B*, but the mean values (±S.E.M.) for between four and twelve different cells.

then decreased and eventually was absent at more positive potentials as the membrane potential approaches the equilibrium potential for Ca^{2+} (E_{Ca}). Such a U-shaped inactivation curve has been interpreted as evidence for a Ca^{2+}-dependent inactivation mechanism (see e.g. Eckert & Chad, 1984). In some tissues, however, the inactivation curve does not return to unity at large positive potentials. This may be explained by the presence of a voltage-dependent component of inactivation in addition to the Ca^{2+}-dependent component (see e.g. Brown, Morimoto, Tsuda & Wilson, 1981), or by Ca^{2+} entry during the tail of current on repolarization during the interval between the pre- and test pulse (Standen & Stanfield, 1982). Pre-pulses to potentials more negative than the holding potential had no effect on the Ca^{2+} current. In early experiments a transient inward current was often observed with negative pre-pulses but it was abolished by tetrodotoxin (TTX) in the bath solution suggesting that it was a voltage-dependent Na^+ current (Plant, 1987*a*, 1988). The

extent of inactivation in two-pulse experiments was very variable from cell to cell and largely independent of the absolute amplitude of the current during the pre-pulse. If pulse protocols were repeated after some run-down, the extent of inactivation did not depend on the absolute amplitude of the pre-pulse current. Some cells with large currents displayed less inactivation than others with small currents. This was also seen in the extent of inactivation in current records with single pulses.

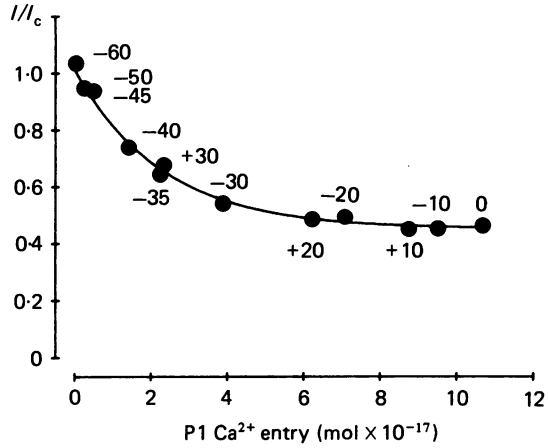

Fig. 4. Ca^{2+} current inactivation as a function of Ca^{2+} entry. The inactivation parameter (I/I_c), calculated as in Fig. 3, is plotted against Ca^{2+} entry during the pre-pulse, measured by integrating the leak-corrected pre-pulse current at the potentials indicated (mV) for one cell. The continuous line is a fit to a single exponential function plus a constant.

Measurement of Ca^{2+} entry

The section above described inactivation as a function of pre-pulse potential. However, if inactivation is dependent on Ca^{2+} entry, the extent of the reduction of the current during a test pulse should depend on the amount of Ca^{2+} entering the cell during the conditioning pulse, independent of the membrane potential. This could explain the large inactivation at +10 mV where the peak inward current during the pre-pulse is normally smaller (e.g. Fig. 1*A*) but inactivation slower, thus allowing a similar amount of Ca^{2+} entry to that during larger currents which inactivate more rapidly. The amount of Ca^{2+} entering the cell during the pre-pulse can be calculated from the leakage-corrected current by estimating the amount of charge entering the cell during the current (the time integral of the current) and dividing by zF, where z is the charge on the ion and F the Faraday constant. In Fig. 4 the inactivation parameter (I/I_c) is plotted as a function of Ca^{2+} entry. In the example in Fig. 4, and in other cells, inactivation increased as Ca^{2+} entry increased. It is noticeable that the relationship between Ca^{2+} entry and inactivation is largely independent of the membrane potential, e.g. in Fig. 4 the values of the inactivation parameter with pre-pulses to +30 and −35 mV (potentials at which the amount of Ca^{2+} entry was nearly equal) were similar despite the large difference in membrane potential. This is in contrast to tissues where a significant voltage-dependent component of inactivation exists and inactivation is greater with pre-pulses on the ascending branch of the

current-voltage relation than on the descending branch despite equal Ca^{2+} entry (Mentrard, Vassort & Fischmeister, 1984). Inactivation does not increase linearly with Ca^{2+} entry but saturates. Test-pulse Ca^{2+} entry calculated by integrating the leak-corrected test-pulse current showed a similar dependence on pre-pulse Ca^{2+} entry, though was less marked because of the large steady-state non-inactivating current.

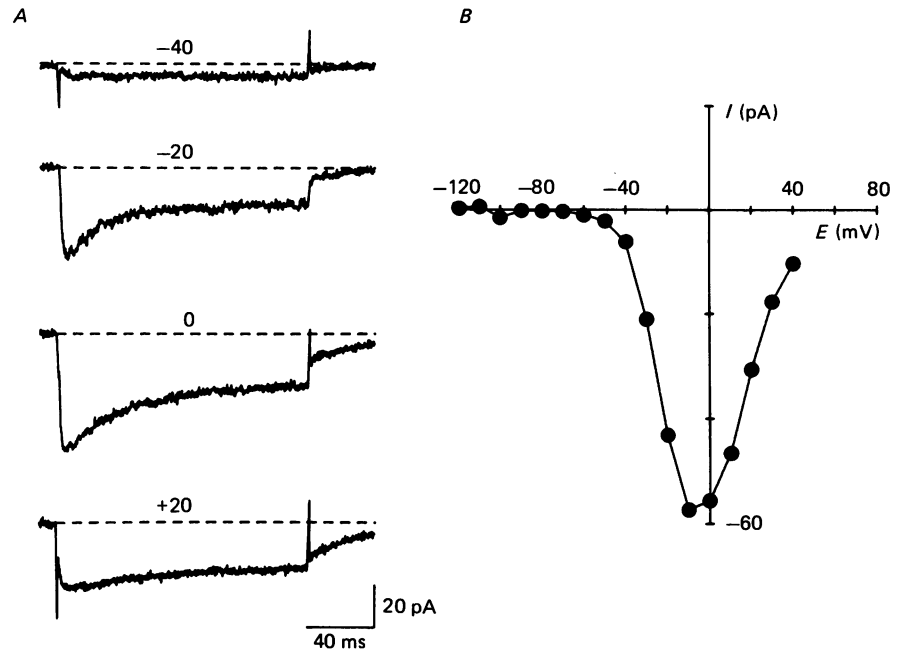

Fig. 5. Effects of BAPTA on Ca^{2+} currents. *A*, currents recorded at the potentials (mV) indicated during step depolarizations from −70 mV with a pipette solution containing BAPTA as a Ca^{2+} buffer. Note the large slow component of the tail current which was not observed with EGTA buffers. *B*, current–voltage relation for inward currents with a BAPTA buffer.

Effects of BAPTA on inactivation

As shown above, considerable inactivation occurs in cells dialysed with high concentrations of EGTA. However, with Ca^{2+} buffers such as EGTA, the internal $[Ca^{2+}]$ does not always correspond to $[Ca^{2+}]$ in the dialysate (Byerly & Moody, 1984), and additionally there may be problems with diffusion and chelation of ions in a thin submembrane layer (Marty & Neher, 1985; Neher, 1986). BAPTA is approximately 5 times more efficient than EGTA (Marty & Neher, 1985), a property attributed to its higher Ca^{2+}-binding rate (Tsien, 1980; Neher, 1986).

If the rate-limiting factor in the inactivation process is the binding of calcium to the chelator, the use of a more rapid chelator should slow inactivation. To test this, pipettes were filled with a BAPTA-Ca buffer with a similar free $[Ca^{2+}]$ and equilibrium Ca^{2+} buffering capacity to the EGTA solution (see Methods). With BAPTA, currents of similar amplitude to those with EGTA-buffered solutions were recorded. Inactivation of currents with BAPTA was somewhat slower (Fig. 5) but

not remarkably different from that with EGTA. One noticeable effect of BAPTA, which was not further investigated, was the larger amplitude of the slow component of the Ca^{2+} tail current.

Effects of the permeating cation

If Ca^{2+} causes inactivation by binding to a site, either within the channel or on another molecule which influences the inactivation of the channel, then other ions may bind less readily and be thus less effective in producing inactivation.

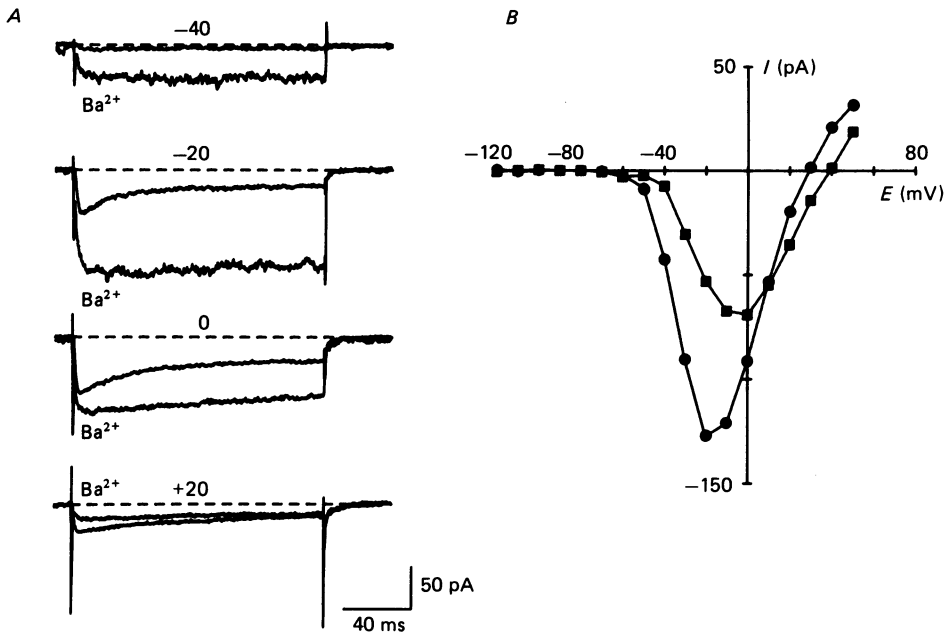

Fig. 6. Currents recorded in Ca^{2+} and following replacement of Ca^{2+} in the bath solution by Ba^{2+} at the potentials (mV) shown, *A*, and the corresponding current–voltage relations in Ca^{2+} (■) and Ba^{2+}(●), *B*.

Barium. Currents through L-type Ca^{2+} channels are larger and show less inactivation when Ba^{2+} is the charge carrier (Lee, Marban & Tsien, 1985) whereas T-type Ca^{2+} channels do not discriminate between Ca^{2+} and Ba^{2+} (Carbone & Lux, 1987; Fox *et al.* 1987*a*). Equimolar replacement of Ca^{2+} by Ba^{2+} had a number of effects on the currents through Ca^{2+} channels. Ba^{2+} currents activated at more negative potentials owing to a shift of the current–voltage relation by 10–15 mV to more negative potentials (Fig. 6*B*). This is most probably an effect on surface potential where Ba^{2+} is less effective at screening or binding to negative surface charges. The maximum inward current in Ba^{2+} was 1·6 times larger than that in Ca^{2+} ($1{\cdot}59 \pm 0{\cdot}13$, $n = 4$) and was shifted to a similar extent to the negative resistance branch of the current–voltage relation. Ba^{2+} currents in contrast to Ca^{2+} currents did not inactivate at potentials < 0 mV and only slightly at more positive potentials (Fig. 6*A*). At all potentials inactivation was slower in Ba^{2+} than in Ca^{2+}.

Sodium. Under normal conditions, in the presence of Ca^{2+} and Mg^{2+}, an

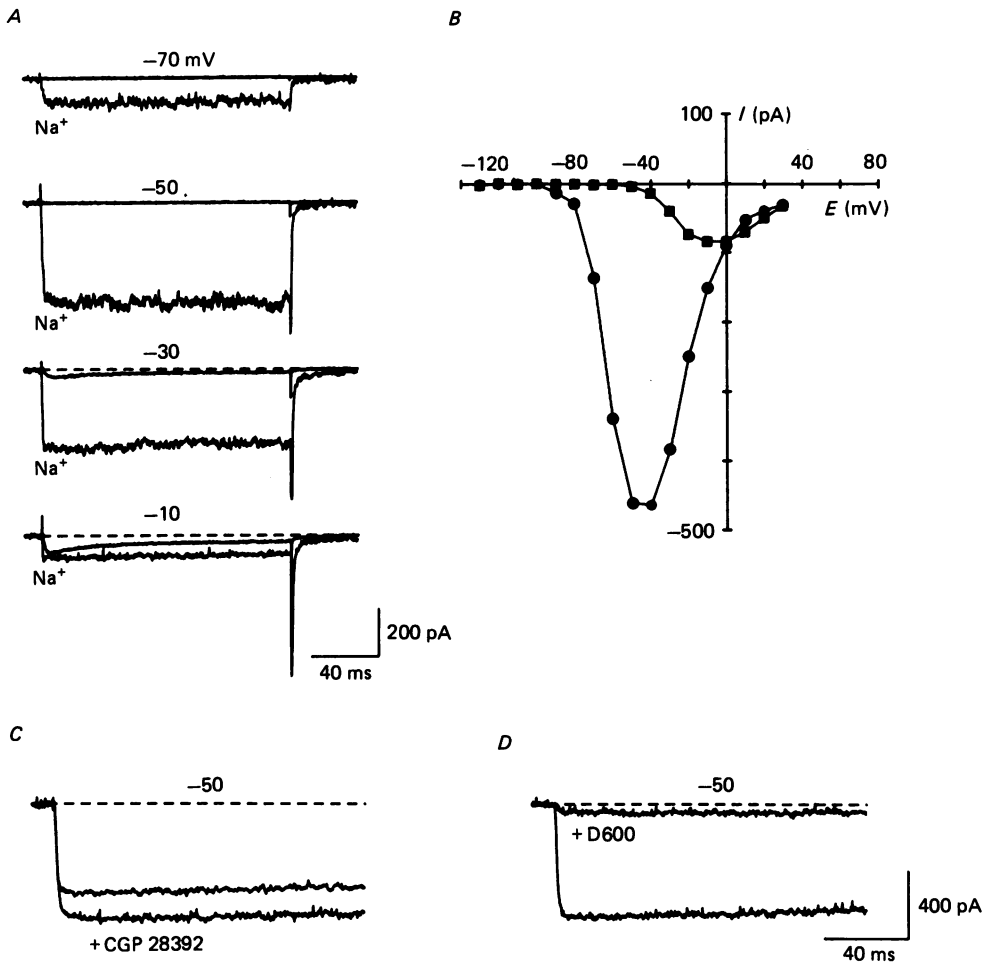

Fig. 7. Effects of divalent cation-free solutions on currents through Ca^{2+} channels. *A*, Ca^{2+} currents recorded in 10·4 mM-Ca^{2+}, and Na^+ currents through Ca^{2+} channels (Na^+) measured following replacement of the bath solution by a Ca^{2+}- and Mg^{2+}-free, EGTA-containing solution. *B*, current–voltage relations in Ca^{2+}-containing (■), and divalent cation-free solutions (●). *C*, increase in the monovalent cation current by CGP 28392 (5 μM). *D*, block of the monovalent cation current by D600 (30 μM). Holding potential, −120 mV.

insignificant proportion of the current through Ca^{2+} channels is carried by monovalent cations such as Na^+. However, when the external divalent cation concentration is reduced to submicromolar levels, monovalent cations may permeate through Ca^{2+} channels. This has been demonstrated in a number of tissues including molluscan neurones, skeletal muscle, cardiac cells and mammalian neurones (Kostyuk & Krishtal, 1977; Almers, McCleskey & Palade, 1984; Hess & Tsien, 1984; McCleskey & Almers, 1985; Fukushima & Hagiwara, 1985; Matsuda, 1986; Carbone & Lux, 1987). In heart cells and smooth muscle cells the inactivation of monovalent cation currents through Ca^{2+} channels has been used as an argument for the co-existence of voltage-dependent and Ca^{2+}-dependent inactivation processes in these

tissues (Lee & Tsien, 1982; Hess & Tsien, 1984; Jmari, Mironneau & Mironneau, 1987).

The inactivation properties of Ca^{2+} channels were compared in 10·4 mM-Ca^{2+} and following the application of a Ca^{2+}- and Mg^{2+}-free solution containing 2 mM-EGTA. In the divalent cation-free solution an inward current developed at a holding potential of -70 mV shortly after applying the solution. The holding potential was therefore changed to potentials < -100 mV, where no inward current was observed. From these holding potentials, voltage steps to potentials less negative than -90 mV activated inward currents (Fig. 7*A* and *B*). The maximum inward current was between -50 and -40 mV, a shift probably owing to the change in surface potential in the absence of divalent cations. These currents which were most likely carried by Na^+ but also possibly by TEA^+ (see also McCleskey & Almers, 1985) were 4 to 5 times larger than the maximum Ca^{2+} current in the same cells. During 150 ms pulses the currents did not significantly inactivate at any potential suggesting that Na^+ is unable to produce inactivation and that no voltage-dependent inactivation contributes to the decline of currents during 150 ms depolarizations. To confirm that these currents flow through the same channels as Ca^{2+} currents, agents known to affect these channels were applied. The dihydropyridine CGP 28392 (5 μM) increased monovalent cation currents (Fig. 7*C*), and the phenalkylamine, D600 (30 μM) blocked most of the current (Fig. 7*D*). Monovalent cation currents were much more stable i.e. they appeared to show less run-down than Ca^{2+} currents in the same cells. On removal of the divalent cation-free solution, normal Ca^{2+} currents could be recorded.

DISCUSSION

The results from whole-cell experiments show that in cultured normal mouse B-cells Ca^{2+} currents inactivate by a Ca^{2+}-dependent process and that only one type of Ca^{2+} channel is present in these cells.

Ca^{2+}-dependent Ca^{2+} current inactivation

In all B-cells investigated, Ca^{2+} currents inactivated with time. A number of observations suggest that inactivation of the Ca^{2+} current is mainly dependent on Ca^{2+} entry. These include the U-shaped inactivation curve, the dependence of inactivation on Ca^{2+} entry during pre-pulses, the change in the rate of inactivation when other cations replace Ca^{2+} as the charge carrier and when $[Ca^{2+}]_o$ is changed, and the dependence of the rate of inactivation on the amplitude of the inward current. However, inactivation of currents at more positive potentials (> -10 mV) was slower than that of currents at more negative potentials (< -10 mV). This would not be predicted if inactivation depends simply on the total amount of Ca^{2+} entering the cell and on the rise in total $[Ca^{2+}]_i$ (Standen & Stanfield, 1982; Chad *et al.* 1984). The slowing of inactivation with larger depolarization has also been observed in *Aplysia* neurones (Chad *et al.* 1984) and in heart cells (Bechem & Pott, 1985), though the effects in these other tissues were not as marked as those observed here. In some cells, no time-dependent inactivation was observed with large depolarizations. Chad & Eckert (1984) suggested that Ca^{2+} may accumulate in local regions or 'domains' associated with individual active Ca^{2+} channels. The local

change in $[Ca^{2+}]$ in a small region near the channel mouth rather than the total bulk free $[Ca^{2+}]$ within the cell may thus influence the properties of the channel. Such a possibility becomes more important when the density of channels is relatively low, as it is in B-cells, and neighbouring channels are less likely to influence each other. The slowing of inactivation with potential is predicted by the model of Chad & Eckert (1984). At more positive potentials, a larger number of channels are activated with a small single-channel current (i). There is thus a smaller rise in $[Ca^{2+}]$ in the domain associated with each channel than at more negative potentials where the number of channels activated is small but i is large. At positive potentials, i decreases non-linearly with potential owing to the rectification caused by the large difference between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ (Hagiwara & Byerly, 1981). $[Ca^{2+}]_i$ is so low that Ca^{2+} can carry little outward current. The rectification of the i–V relation could explain the increase in the rate of inactivation between 1 and 10 mM-Ca^{2+} in Fig. 2, where in 10 mM-Ca^{2+} the number of Ca^{2+} channels activated is smaller, owing to a shift of the activation parameters to more positive potentials, but i is larger because of the increase in $[Ca^{2+}]_o$.

An alternative possibility for the slowing of inactivation at more positive potentials is that the properties of Ca^{2+} channels are changed and possibly their sensitivity to Ca^{2+} reduced. A second open state would explain the change in kinetics of the B-cell Ca^{2+} current with larger depolarizations without the necessity of assuming a Ca^{2+} dependence. However, the results of two-pulse experiments, where the potential dependence of inactivation is similar to that of Ca^{2+} entry and the dependence of inactivation on the amount of Ca^{2+} entering the cell is independent of the potential of the pulse producing Ca^{2+} entry, are less readily explained by two open states. A slow voltage-dependent component of inactivation in the B-cell cannot be excluded since pulses longer than 200 ms were not used.

Ca^{2+} channel sub-types in B-cells?

It has been suggested that normal B-cells may have a number of types of Ca^{2+} channels to account for the Ca^{2+} dependence of the spikes and the slow waves and to explain the effects of Ca^{2+} channel agonists on glucose-induced electrical activity; for instance, CGP 28392 increases the amplitude and duration of the plateau but inhibits spike activity (Henquin, Schmeer, Nenquin & Meissner, 1985). A similar effect on the spike activity is seen when $[Ca^{2+}]_o$ is increased probably due to an increase in Ca^{2+} -dependent inactivation of Ca^{2+} channels and activation of K^+ channels (Meissner & Schmeer, 1981). The changes in the Ca^{2+} current with CGP 28392 can explain its effects on electrical activity and the increase in insulin release (Henquin *et al.* 1985; Malaisse, Sener & Malaisse-Lagae, 1985). The effects of higher concentrations of nifedipine on electrical activity, a transient inhibition followed by a decrease in spike frequency (Vasseur, Debuyser & Joffre, 1987), may be explained by the block of the Ca^{2+} current.

The results obtained in this paper can be explained on the basis of a single type of Ca^{2+} channel present in the B-cell membrane. This is supported by evidence from the voltage dependence, kinetics, pharmacology, inactivation properties, and behaviour with other permeant cations. All of these observations are consistent with the properties of L-type Ca^{2+} channels and not with those of T- or N-type channels (see

Fox *et al.* 1987*b* for a summary of the properties of the channel types). In other work on normal mouse B-cells (Rorsman & Trube, 1986) and neonatal rat B-cells (Satin & Cook, 1985), only a single type of Ca^{2+} channel has been observed. The situation in tumour cells is not as clear. Findlay & Dunne (1985) described a rapidly activating and inactivating current in RINm5F cells which had some of the characteristics of a Ca^{2+} current. Another group suggested that this current is a voltage-dependent Na^+ current, since it was blocked by TTX, and in addition saw a more slowly activating Ca^{2+} current similar to that in normal B-cells (Rorsman *et al.* 1986). Recent single-channel channel evidence from RINm5F cells indicates that two types of Ca^{2+} channels are present in these tumour cells which may correspond in their voltage dependence, conductance and pharmacological properties to T- and L-type channels (Velasco, 1988).

Significance of the Ca^{2+} current inactivation and selectivity

As already described by Rorsman & Trube (1986), the Ca^{2+} currents seen in voltage-clamp experiments in single cultured B-cells correspond to the channels involved in glucose-induced electrical activity. The activation range of the channels corresponds to the potentials where slow wave and spike activity occur, and the channels are influenced by substances which change the pattern of electrical activity. Ca^{2+}-dependent Ca^{2+} current inactivation will certainly play a role, together with Ca^{2+}-dependent K^+ channels, in the negative-feed-back control of Ca^{2+} entry in the B-cell. Henquin (1980) suggested that Ca^{2+} itself and depolarization are involved in the inactivation of the Ca^{2+} uptake mechanism in B-cells from experiments studying the effects of tolbutamide on insulin release. It is probable that at the resting $[Ca^{2+}]_i$, which from Ca^{2+} indicator experiments is $\sim 1{\cdot}5 \times 10^{-7}$ M (see e.g. Rorsman & Abrahamsson, 1985), a number of Ca^{2+} channels are already inactivated. In molluscan neurones the dissociation constant (K_d) for the effect of internal Ca^{2+} on Ca^{2+} channels is around 7×10^{-7} M (Plant, Standen & Ward, 1983). It is not possible to determine how high the $[Ca^{2+}]$ rises in the submembrane compartment in the B-cell in these experiments, but inactivation will most probably have a role in the range of $[Ca^{2+}]_i$ in the B-cell at rest and during glucose-induced electrical activity. However, it should be borne in mind that the changes in B-cell metabolism, which occur upon stimulation by glucose or other fuel insulin secretagogues, might modulate the inactivation of Ca^{2+} currents.

The large Na^+ inward current at negative membrane potentials observed in the absence of extracellular divalent cations plus EGTA will be sufficient to depolarize the B-cell membrane. It might thus account for the depolarization of the B-cell membrane that occurred upon omission of Ca^{2+} and Mg^{2+} in the absence of EGTA (Atwater & Beigelman, 1976).

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