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Studies of calcium-dependent ionic currents and their muscarinic regulation in pancreatic insulin-secreting cells

by

J. Ashot Kozak

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfilment of the requirements for the degree of Doctor of Philosophy,

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Abstract

Studies of calcium-dependent ionic currents and their muscarinic regulation in pancreatic insulin-secreting cells

by

J. Ashot Kozak

Preceptor: Dr. Diomedes E. Logothetis

Ca^{2+}-dependent Cl^- and K^+ currents were characterized in insulin-secreting βTC-3 cells using perforated-patch whole cell recording technique. Both Ca^{2+}-dependent currents were found to be activated by depolarizing voltage steps which allowed influx of Ca^{2+} through the Ca^{2+} channels. The Ca^{2+} channels in βTC-3 cells were also described and found to belong to the L-type family based on its kinetic and pharmacological properties, similar to those found in native β cells. The Ca^{2+}-dependent currents activated slowly and had characteristic tail currents revealed upon repolarization. The Ca^{2+}-dependent chloride current reversal potential was sensitive to changes in Cl^- concentrations in the bathing solution and Br^-, NO_3^-, I^- and acetate-were permeant anions in addition to Cl^-. Niflumic acid and DIDS were effective blockers of this current at high micromolar concentrations. The Cl^- ion equilibrium potential was found to be

close to -22 mV under conditions where the internal Cl^- concentration was undisturbed. Both Ca^{2+}-dependent currents could also be activated if Ca^{2+} in the extracellular medium was substituted with Sr^{2+}. Ba^{2+}, however was not effective in activating these currents. The Ca^{2+}-dependent K^+ current exhibited unique pharmacological properties: it was sensitive to block by charybdotoxin and clotrimazole and quinine but not tetraethylammonium, apamin, iberiotoxin, scyllatoxin and kaliotoxin. This current was found to be activated by niflumic acid. The Ca^{2+}-dependent K^+ channels were permeable to Tl^+, Rb^+ and NH_4^+ but not to Cs^+ and Na^+. A current which was Ca^{2+}-dependent and displayed kinetics similar to the βTC-3 K^+ current was present in porcine islet cells. It was blocked by quinidine but was insensitive to charybdotoxin.

Muscarinic stimulation of the βTC-3 cells was capable of activating both Ca^{2+} dependent currents in the presence or absence of external Ca^{2+}, presumably by releasing Ca^{2+} from internal stores. Both currents, when activated by muscarinic agonists often displayed time dependent oscillations. Thus, the ionic mechanism underlying muscarinic regulation of the insulin secreting cells has been described.

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Chapter 1

Background

Glucose-induced electrical activity

Pancreatic β-cells, residing in the endocrine portion of the pancreas, secrete insulin in response to plasma glucose level elevation. β-cells have been shown to exhibit electrical activity in response to various secretagogues like glucose, amino acids and acetylcholine. Generally, glucose and amino acids are referred to as fuel secretagogues. Meissner (Meissner 1976) showed that increase in the extracellular glucose causes a biphasic electrical response in the β-cell: prolonged depolarizations with superimposed action potentials (plateau phase) interrupted by silent phases where the cell does not fire and is hyperpolarized (Fig.1). Early on the question arose whether the glucose-induced electrical activity is directly related to insulin release. It was shown for example (see Meissner & Schmelz 1974; Meissner 1976), that the increase in glucose concentration dose-dependently increases the spike frequency and plateau phase duration. It was known that incremental increases in glucose concentration caused increased insulin secretion from the isolated β-cells. The burst activity in β-cells occurs only at glucose concentrations which also cause insulin release. The potential levels of the electrical activity are more or less independent of the glucose concentration.

Fig. 1. Regular glucose-induced electrical activity of a single β-cell. The upper part shows a train of slow waves in the presence of 10 mM glucose. Record (a) shows the three slow waves marked by a bar in the upper part at an expanded time scale. The recording was done using intracellular recording with a sharp electrode. (From Meissner, 1992).

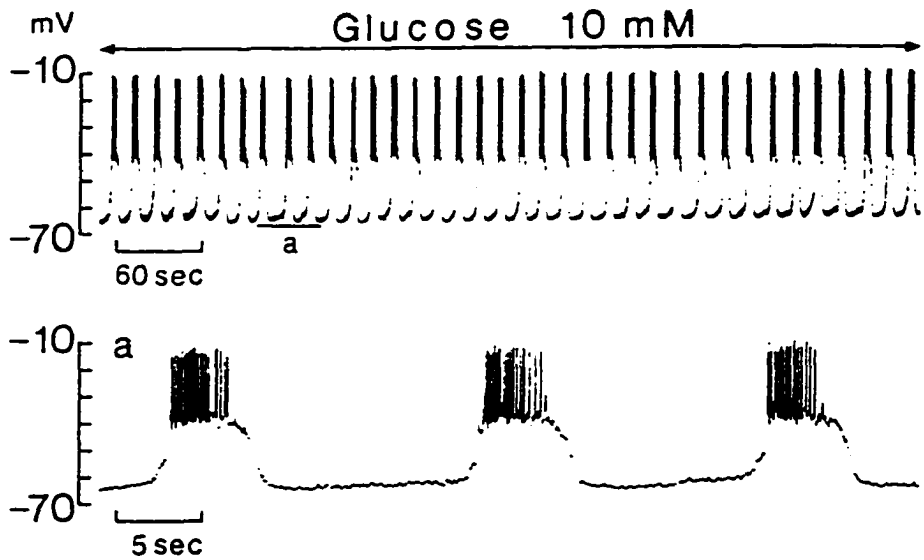


Fig. 1

(Dean and colleagues (Dean, Matthews & Sakamoto 1975) on the other hand had shown that a linear relationship existed between membrane potential and the log of glucose concentration in the range of 1-30 mM glucose). Whereas the membrane potential is not glucose dependent, the time course of the burst activity reveals a marked dependence on glucose concentration. Thus, with increasing concentration of glucose, the duration of the spiking plateau phase becomes longer. In very high glucose concentrations (>16 mM), the bursting pattern disappears altogether and the membrane remains depolarized at the plateau potential (Beigelman & Ribalet 1980; Ashcroft & Rorsman 1991). This results in continuous spike activity. When the glucose level is lowered to substimulatory levels (glucose concentration that does not stimulate insulin release) the electrical activity disappears and the membrane hyperpolarizes markedly. In 2.8 mM glucose the membrane potential is around -60 mV. At substimulatory glucose concentrations, where there is only basal insulin release, an increase in the external glucose causes only a depolarization of the membrane, however no electrical activity in the β-cells is seen.

Bursting activity is commonly regarded as the major activity by which the β-cell imports Ca^{2+} from the extracellular space. Glucose-induced electrical activity in β-cell shows several characteristics: there are bursts of action potentials which last 3-4 seconds. They are generally followed by a silent phase or post-burst hyperpolarization which may last as long as 10 s. During this hyperpolarized phase the membrane potential slowly depolarizes and brings the membrane close to the threshold potential for firing. One difference

from neurons is that the action potentials do not occur from a hyperpolarized resting potential but rather from a plateau potential. The plateau potentials are generally around -35 mV. The hyperpolarized (silent phase) potentials are around -45 to -50 mV. The spike amplitude is ≈15 mV. During a burst the spikes vary considerably in amplitude, duration and shape. In some cells the maximal amplitude of the spike can reach up to 30 mV. The ionic currents underlying the single action potential (spike) which appears superimposed on the plateau have been elucidated. The action potential lasts approximately 0.2 seconds. The rising phase is mediated by L-type (dihydropyridine (DHP)-sensitive) calcium channels whereas the hyperpolarization is mediated by a tetraethylammonium (TEA) sensitive delayed rectifier current. The sodium current seems not to have a role in the action potential since at the plateau potential most of it should be inactivated. Also, additions of tetrodotoxin (TTX) to the medium do not affect the firing pattern of the β-cell.

The situation in canine islet cells is different, however. Misler's group (Pressel & Misler 1991) showed that in dog islet cells the rising phase of the action potential is actually mediated by the Na^+ channel. Rodent β-cells routinely demonstrate the presence of a Na^+ current (Plant 1988, J. A. K. unpublished). The function (if any) of these currents remains obscure.

A rise in $[Ca^{2+}]_i$ resulting from the influx through the β-cell plasma membrane is the primary trigger for glucose-induced insulin secretion. The bursting electrical activity in response to glucose ceases when external Ca^{2+} is

removed (Meissner & Schmelz 1974). Removal of external Ca^{2+} also stops insulin secretion. This strongly suggests a correlation between electrical activity and insulin secretion. The membrane potential stabilizes at approximately 4 mV depolarization measured from the potential level attained during the silent intervals. Under these conditions another sort of spikes occur erratically and spontaneously. These spikes resemble the action potentials seen in other excitable cells to a greater degree. They arise from a constant potential level and end with a marked hyperpolarization. The ionic basis of the spikes in the absence of external Ca^{2+} remains unclear. They resemble genuine action potentials but occur only in the potential range between the repolarization and plateau level of the bursts. The pronounced afterhyperpolarization may be due to an increased K^+ permeability in analogy to neurons. It is not clear if these spikes are linked to insulin release. Another explanation for these spikes may lie in the degree of electrical coupling of the cells when Ca^{2+} is present or absent. The basal secretion of insulin at substimulatory glucose concentrations cannot be explained by the studies of Meissner and colleagues however.

Voltage-gated Ca^{2+} channels have been described in various β-cell preparations and insulin-secreting cell lines (reviewed in Ashcroft & Rorsman 1991). It is evident that β-cells possess a heterogeneity of Ca^{2+} channels. There are also species differences. Thus, mouse β-cells express L-type (DHP-sensitive) channels whereas rat β-cells have both L and T type Ca^{2+} channels.

The T-type Ca^{2+} channel is low voltage activated (Hess 1990), and can open at potentials as negative as -50 mV. It also exhibits faster inactivation. Single-channel recordings demonstrated that L-type channel openings occur close to the β-cell resting potential. This suggests that Ca^{2+} influx through these channels may contribute to the background Ca^{2+} influx into the cells. This would explain the observation that DHPs modulate basal insulin secretion (Boschero, Carroll, De-Souza & Atwater 1990). There may be more than one type of high voltage activated (HVA, L-like) Ca^{2+} channels in β-cells. In RIN-5F (rat insulinoma) cells for example, DHPs do not block the total HVA current. This argues in favor of existence of a DHP-insensitive L-like current. (Pollo, Lovallo, Biancardi, Sher, Socci & Carbone 1993). The function of T-type channels in β-cell Ca^{2+} influx is not known.

The discovery of the cyclic changes of cytoplasmic Ca^{2+} in the pancreatic β-cells (Santos, Barbosa, Silva, Antunes & Rosario 1992; Lund & Hellman 1992 -1993; Rosario, Barbosa, Antunes, Silva, Abrunhosa & Santos 1993; Eberhardson, Tengholm & Grapengiesser 1996) raises the question of how the oscillatory pattern is related to the ability to recognize glucose as a secretory stimulus. There is evidence that some cellular functions may be regulated by amplitude or frequency modulations of $[Ca^{2+}]_i$ oscillations. The insulin concentrations also oscillate cyclically. It has been demonstrated that $[Ca^{2+}]_i$ oscillates in the glucose-stimulated cell with a similar frequency as the pulsatile insulin release. The slow sinusoidal oscillations (called type A) are

those which most closely correspond to pulsatile insulin release. Concentration of glucose is a determinant for their generation and further transformation into a sustained increase, it does not affect the properties of type A oscillations in individual β-cells.

The ionic mechanisms underlying the burst activity remain unknown (Ashcroft, Proks, Smith, Ämmälä, Bokvist & Rorsman 1994; Rorsman, Bokvist, Ämmälä, Eliasson, Renström & Gäbel 1994), although a number of ion channels have been characterized in β-cells over the past years using the patch-clamp technique. One aspect of β-cell electrical activity is well understood. In the absence of external glucose the resting potential of the β-cell (-70 mV) is determined by the activity of the ATP-sensitive K^+ channel. Glucose application depolarizes the cell by increasing the ATP/ADP ratio as a result of glucose metabolism. This causes the closure of the K-ATP channels. There must exist an inward current which counteracts the outward K-ATP current. In the absence of K-ATP channels this current should bring the equilibrium potential to more depolarized values. It may be carried either by Na^+ or Cl^- or both. The nature of this resting conductance is unknown. It was shown however, that replacement of external Na^+ with an impermeant cation N-methyl D-glucamine causes a repolarization, cessation of firing activity and lowering of internal Ca^{2+}. The initial slow depolarization thus comes about because of closure of K-ATP channels. In the presence of tolbutamide (a specific K-ATP channel blocker) the bursting of the β-cell switches into

continuous firing (Rosario et al. 1993). This originates from suppression of the K-ATP channel which governs the resting potential of the β-cell.

Several studies (Sehlin 1978; Eddlestone & Beigelman 1983; Henquin & Meissner 1984; Sehlin & Meissner 1988) have addressed the role of Cl^- ions in determining the electrical activity of the β-cell. For example, in a study by Sehlin and Meissner (1988), external Cl^- was substituted by other anions and the effects on the bursting pattern examined. Reduction of external Cl^- resulted in shifting the repolarization potential to more positive values and a transient increase in the plateau fraction. This result implicates Cl^- conductances in the bursting activity induced by glucose. The nature of these conductances is not elucidated.

A model explaining the bursting activity was proposed by Atwater and colleagues (Ribalet & Beigelman 1979; Atwater, Dawson, Scott, Eddlestone & Rojas 1980; Ribalet & Beigelman 1980; Atwater, Rosario & Rojas 1983; Sherman 1996). According to this model the β-cell bursting activity has been attributed to a feedback loop involving a large-conductance $I_{K(Ca)}$ current and $[Ca^{2+}]_i$. However, more recently this model has been challenged. It was shown (Kukuljan, Goncalves & Atwater 1991) that the Maxi K(Ca) channel is not active within the range of membrane potentials where bursting occurs. A distinct Ca^{2+}-dependent K^+ channel has been implicated in the control of the β-cell membrane potential Ämmälä, Bokvist, Larsson, Berggren & Rorsman

1993. Its possible involvement in the regular glucose-induced β-cell bursting activity remains unknown till now (see *Discussion*).

One more recent study (Rosario et al. 1993) addressed the issue of the mechanism underlying the bursts. A number of agents were used which would increase cytoplasmic Ca^{2+} concentration: ionomycin (a Ca^{2+} ionophore), high external Ca^{2+}, Bay K (a dihydropyridine). Ionomycin, increased external Ca^{2+}, and Bay K all evoked bursting activity in a β-cell in the presence of tolbutamide. All these agents increasing Ca^{2+} influx can restore bursting activity in the presence of tolbutamide. Charybdotoxin (a peptide isolated from scorpion venom, Miller, Moczydlowski, Latorre & Phillips 1985) was used to test whether the K(Ca) (TEA-sensitive) current has any effect on the burst activity. 40-80 nM ChTX failed to affect the bursting activity evoked by 12 mM Ca^{2+}. The authors ascribe this to the lack of effect of the Maxi K(Ca) channel on the bursts. 200 μM quinine also did not affect the bursting activity of the β-cell. It should be noted that bursting activity seldom occurs in isolated normal β-cells, mostly it is seen in intact islets (Rorsman et al. 1994).

Recent theoretical studies (Sherman 1996; Mears, Sheppard, Atwater, Rojas, Bertram & Sherman 1997), addressing the question of the plateau phase depolarization have suggested that it may arise from an inward nonselective cationic current, called I_{CRAN}. This current is thought to be activated as the intracellular Ca^{2+} stores empty and is deactivated as they refill. It is distinguished from its counterpart in non-excitable cells, the I_{CRAC},

which is Ca^{2+}-selective. The experimental evidence for this current's existence remains circumstantial, however.

Aside from glucose, the chief stimulus, insulin secretion can also be modulated by amino acids. In the presence of amino acids 5 mM glucose is able to elicit an electrical activity whereas in their absence 5 mM glucose only slightly depolarizes the cell membrane (Bolea, Pertusa, Martin, Sanchez-Andres & Soria 1997). Generally the presence of amino acids shifts the glucose concentration curve leftward.

Ion channels in β-cells

In this section we will describe some of the ion channels studied in detail in insulin-secreting cells.

K-ATP channel

K-ATP channels have been identified in β-cells in various species: rodent, canine and human (Barnett & Misler 1994). The β-cell K-ATP channel belongs to the same family of ATP-inhibited channels also found in cardiac, smooth and skeletal muscle cells (Takano & Noma 1993). The current-voltage relation for this current shows pronounced inward rectification. The rectification results primarily from a voltage-dependent block by internal Mg^{2+} and polyamines (Lopatin, Makhina & Nichols 1994). In symmetrical high K^+ the conductance

for the inward current is found to be 50-75 pS. In normal external K^+, the conductance for outward current is 20-30 pS (Misler, Falke, Gillis & McDaniel 1986; Bokvist, Rorsman & Smith 1990). The K-ATP channels are not permeable to Na^+. Rb^+ permeability is comparable to K^+ permeability (P_{Rb}/P_K=0.7 (Takano & Noma 1993)). The channel openings have a characteristic feature in that they open in bursts of variable duration, followed by periods of inactivity. The main effect of ATP is to reduce the burst length and increase the duration of closed intervals between the bursts. In the cell-attached configuration glucose and tolbutamide (a specific blocker) have a similar effect on the channel activity (Gillis, Gee, Hammoud, McDaniel, Falke & Misler 1989). There is evidence that the K-ATP channel can be activated by G-proteins in RIN-5F cells. In the presence of Mg^{2+}, GTPγS, a non-hydrolysable GTP analogue, produces an increase in channel activity (Findlay 1987; Dunne & Petersen 1991). This may mean that the channel can be regulated by hormones under physiological conditions. Very specific drugs are available to block and activate the K-ATP channel. The best known are the drugs of sulphonylurea family. The blockers glibenclamide and tolbutamide are examples. In β-cells it is demonstrated that tolbutamide is specific for the K-ATP channel as it does not block other K^+ channels (Rorsman &Trube 1986). Sulphonamide drug diazoxide serves as a potent opener of the K-ATP channel.

Delayed recifier K^+-channels

Delayed rectifying K^+ (K-DR) currents have been found in rodent and human β-cells (Rorsman & Trube 1986; Kelly, Sutton & Ashcroft 1991), as well as RIN and HIT cell lines (Fatherazi & Cook 1991). Whole cell K-DR currents are activated at voltages above -30 mV and increase linearly with increased depolarization. When K-DR currents are recorded in high external K^+ solutions, they start activating at potentials as negative as -50 mV. The time course of activation also slows down noticeably. The K-DR currents inactivate slowly during depolarizing voltage steps lasting several seconds. Under physiological K^+ concentrations the single K-DR channel current-voltage relation shows outward rectification with conductances ranging 8-15 pS. The delayed rectifier channel is strongly potassium selective. P_{Na}/P_K=0.05. (Kelly et al. 1991). TEA blocks the K-DR current in a concentration-dependent manner: at 0 mV 1.4 mM TEA produces 50% inhibition. Micromolar conentrations of quinine also abolish the K-DR current. K-DR is not affected by sulphonylurea drugs glibenclamide, tolbutamide or diazoxide.

Ca^{2+}-activated K^+ channels

The existence of a Ca^{2+}-activated K^+conductance was first hypothesized by Atwater and colleagues (Atwater et al. 1983). Direct evidence for the existence of a K(Ca) channel was provided from patch-clamp studies. Several lines of

evidence were supplied by whole-cell studies of β-cells. The inhibition of Ca^{2+} current by low concentrations of Cd^{2+}, Ni^{2+} or organic antagonists results in reduction of the outward K^+ current (Smith, Bokvist, Arkhammar, Berggren & Rorsman 1990). Removal of extracellular Ca^{2+} decreases the outward current amplitude. The Ca^{2+}-sensitive component of the outward current decays during a prolonged recording with a time course similar to the rundown of Ca^{2+} current. Charybdotoxin inhibits 10-20% of the outward current. In some cells the I-V relation for the whole-cell current shows N-shaped voltage dependence, that can be blocked by Cd^{2+}. The fraction of the outward current dependent on the Ca^{2+} influx is reduced following a long dialysis with EGTA (Satin, Hopkins, Fatherazi & Cook 1989). The K(Ca) current activates rapidly and inactivates completely within 150 ms. This inactivation probably reflects the intrinsic gating of the channel, since Ca^{2+} decay is much slower. The single-channel conductance is 200-250 pS in symmetrical high K^+ and 110-155 pS under physiological K^+ conditions . These characteristics identifiy this channel as Maxi-K(Ca), a channel seen in many other preparations (reviewed in Marty 1983). The Maxi K(Ca) in β-cells is both voltage and Ca^{2+}-activated. Increase in intracellular Ca^{2+} concentration shifts the relationship between open probability and membrane potential towards more negative potentials. This results in more activity at a given potential. In adult β-cells no activity of the Maxi K(Ca) channels is observed at the resting potential in cell-attached patches. Therefore it is unlikely that Maxi K contributes to the resting potential

of the β-cell. As discussed above, the Maxi K(Ca) current is not involved in normal bursting activity induced by glucose (Kukuljan et al. 1991). The Maxi K(Ca) channels are inhibited by quinine and TEA. The sensitivity to TEA is much higher than the K-ATP channel, thus 0.5 mM TEA completely blocks the Maxi K(Ca) in rat cells (Tabcharani & Misler 1989). The block produced by TEA is assymetric, external TEA is 100 times more potent than the internal TEA. At 0.15 mM quinine exhibits half-maximal block. Low nanomolar concentrations block the Maxi K(Ca) channel. In insulinoma cell lines the Maxi K^+ channel is modulated by glucose metabolism (Ribalet, Eddlestone & Ciani 1988). In HIT cells, glucose inhibits the channel dose-dependently which is complete at 25 mM.

Small conductance Ca^{2+}-activated K^+ current

Two articles from P. Rorsman's group have presented evidence that in addition to the Maxi K(Ca) channel, the mouse β-cells also express a novel Ca^{2+}-activated K^+ channel. (Ämmälä, Larsson, Berggren, Bokvist, Juntti-Berggren, Kindmark et al. 1991) showed that oscillatory electrical activity in cultured β-cells can result from transient activation of a K^+ conductance. The current could be activated by intracellular perfusion of GTPγS. They report that in 25 % of the cells periodic activation of this current could be seen without application of intracellular GTP (Ämmälä et al. 1993). The oscillations without

internal GTP were however short lived. This novel K(Ca) current was not blocked by tolbutamide or apamin, a bee venom toxin specific for SK (small conductance) K(Ca) channels found in other tissues (Blatz & Magleby 1986). 5 mM TEA seemed to have a minuscule effect on the current, and it was explained by Maxi K(Ca) block. This current could be also activated by addition of low concentrations of carbachol. Most probably the channel is activated by the phospholipase C pathway resulting in increases in internal Ca^{2+} due to release form IP_3-sensitive stores. From noise analysis this channel was shown to have a conductance of 0.5 pS. The I-V relationship is ohmic over the whole voltage range, suggesting that this channel is not voltage-dependent, unlike the Maxi K(Ca) channel. The authors also found that Ca^{2+} entering the cell via the DHP-sensitive Ca^{2+} channels is paradoxically less effective in activating the K(Ca) channel than Ca^{2+} released from the internal stores. It is likely that this K(Ca) current can be activated by Ca^{2+} influx under certain experimental conditions. The protracted action potentials which are observed in the presence of TEA are associated with large $[Ca^{2+}]_i$ transients reaching up to 1 μM (Rorsman, Ämmälä, Berggren, Bokvist & Larsson 1992). Repolarization during the long TEA-induced action potentials can occur in the presence of tolbutamide. At these potentials the K-DR and Maxi K(Ca) channels are also inactive and that leaves the novel K(Ca) current as the only conductance present. There is no definitive proof of this channel's involvement in the repolarization of the action potential (see *Discussion*).

Muscarinic regulation of the electrical activity and insulin release

The endocrine pancreas is extensively innervated by the parasympathetic nervous system. Parasympathetic activity via the vagus nerve results in increased insulin release and there is a cephalic phase of insulin secretion, which occurs before the entrance of food into the gastrointestinal tract (Woods & Porte 1974). Muscarinic agonists such as carbachol at micromolar concentrations have been shown to increase insulin release (Sharp, Culbert, Cook, Jennings & Burr 1974) and electrical activity (Gagerman, Idahl, Meissner & Täljedal 1978) in β-cells. They act through M_3 muscarinic receptors in β-cells (Verspohl, Tacke, Mutschler & Lambrecht 1990). M_3 receptors are coupled to the phospholipase C pathway, which upon activation results in the generation of inositol 1,4,5-trisphosphate (IP_3) and subsequent release of Ca^{2+} from IP_3-sensitive stores located in the endoplasmic reticulum (Malaisse 1986; Nilsson, Arkhammar, Hallberg, Hellman & Berggren 1987; Weng, Davies & Ashcroft 1993). At glucose concentrations below the threshold for stimulation of insulin secretion and electrical activity, carbachol depolarizes the β-cell and initiates electrical activity and insulin release (Gagerman et al. 1978; Cook, Crill & Porte 1981). At higher glucose concentrations carbachol increases the burst length and reduces the silent

phases (Gagerman et al. 1978). In isolated mouse β-cells (Ämmälä et al. 1991) showed that carbachol application caused repetitive short hyperpolarizations when the recording pipette contained GTPγS. As discussed in the previous section, these hyperpolarizations were attributed to a Ca^{2+}-activated K^+ current. The ionic basis of both the depolarizing and hyperpolarizing muscarinic response is poorly understood. Experiments performed by Henquin and colleagues (Gilon, Nenquin & Henquin 1995) using single islets suggested that the depolarizing action of ACh increases the intracellular Na^+ concentration. Since the rise in internal Na^+ was prevented by its removal from the external solution but not by blocking of the Na^+ pump by ouabain, it was hypothesized that ACh activates a Na^+ conductance in the β-cell. TTX did not affect this [Na^+] rise. In the studies by (Bordin, Boschero, Carneiro & Atwater 1995) and (Sherman 1996) the depolarizing response to muscarinic agonists is explained by activation of the I_{CRAN} upon depletion of the intracellular Ca^{2+} stores by IP_3. The dependence of the depolarizing response on the presence of external Na^+ (as argued by Henquin's group) can be accounted for by the fact that the I_{CRAN} in β-cells is a non-selective channel and allows Na^+ inflow. It should be noted however that the latter studies do not use the substitution of external Na^+ to demonstrate the abolishing of the depolarizing muscarinic response.

The aim of this study was to elucidate the ionic basis of insulin-secreting cell response to muscarinic agonists. It is found that stimulation activates two Ca^{2+}-activated currents- Cl^- and K^+ which have not been described previously.

Chapter 2

Materials and methods

βTC-3 cells

In mammalian pancreas, the β-cells are localized in the islets of Langerhans, which are dispersed throughout this organ (Pipeleers 1987). The islet cells comprise only about 1% of the total pancreas. The limited availability of normal islets and their cellular heterogeneity has led many investigators to develop immortalized β-cell lines as models for studies of β-cell physiology and biochemistry.

Two approaches have been used previously to generate immortalized β-cell lines: one involves induction of an insulinoma by X-ray irradiation, an example of which is the RIN cell line (Gazdar, Chick, Oie, Sims, King, Weir et al. 1980); the other involves infection and transformation of normal islets with simian virus-40 (SV-40), HIT cells were created using the latter method (Santerre, Cook, Crisel, Sharp, Schmidt, Williams et al. 1981). While these cell lines have served well for studying insulin secretion and electrical activity accompanying it, their behavior deviates from that of "normal" β-cells. The RIN cell line releases insulin at abnormally low concentrations of glucose, while in both cell lines the insulin content is only 0.1% of that found in normal β-cells and the insulin content and secretory response to glucose decline with passage number.

Hanahan (Hanahan 1985) demonstrated that the promoter of rat insulin II gene can target expression of the SV-40 large T-antigen specifically to pancreatic β-cells in transgenic mice. These mice frequently develop insulinomas. Efrat and colleagues (Efrat, Linde, Kofod, Spector, Delannoy, Grant et al. 1988) used insulinomas from these transgenic mice to generate cell lines designated βTC. Since in βTC cell lines the immortalizing oncogene is controlled by the insulin gene regulatory region, the βTC lines produce significantly higher levels of insulin than RIN or HIT cells (D'Ambra, Surana, Efrat, Starr & Fleischer 1990). One other advantage is that the cell line can be renewed by primary culture of new insulinomas in the transgenic mice with heritable phenotype.

In βTC-3 cells insulin release is stimulated by lower concentrations of glucose (0.15 mM) than in normal β-cells (2.8 mM) (D'Ambra et al. 1990). Glucose sensing in β-cells requires metabolism of glucose and therefore the dependence of insulin release on glucose concentration parallels the dependence of the glucose utilization on applied glucose concentration. Two rate limiting steps in glucose utilization are the GLUT2 glucose transporter and glucokinase, the enzyme responsible for phosphorylation of glucose. βTC-3 cells express predominantly the GLUT1 transport isotype but their glucokinase activity is comparable to that in "normal" β-cells. As in "normal" β-cells, insulin secretion from the βTC-3 cells is stimulated by a

variety of secretagogues including muscarinic agonists, and is potentiated by glucagon and other agents that increase intracellular cAMP (D'Ambra et al. 1990; Gromada & Dissing 1996). The βTC-3 cells also express the K-ATP channel, a hallmark of β-cells (Fan, Tokuyama & Makielski 1994).

The βTC-3 cell line is a nonclonal cell population. Over the course of this study a great variability was observed in the degree to which a particular channel was expressed. The predominance of a channel expression varied with duration of growth after splitting. No strict correlation could be deduced however. The cells of passages 33-50 were very consistent in their phenotype, in that the frequency of appearance of specific conductances did not change. Overall, it appears, the $I_{Cl(Ca)}$ was seen more frequently than $I_{K(Ca)}$. Whether this is a consequence of selection of a subpoputlation of cells or adaptation of the entire population to the growth conditions is not known.

Cell culture

βTC-3 cells were kindly provided by Drs. N. Fleischer and S. Efrat (Albert Einstein School of Medicine, Bronx, NY). The cells (passages 33-50) were grown in 10 ml plastic dishes (Beckton-Dickinson, Lincoln Park, NJ) containing DMEM (Gibco, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and 12.5 mM glucose and penicillin/streptomycin. At times fungizone was also included in the medium. The cells were fed twice a

week, passaged using 0.05% trypsin/0.53 mM EDTA, and kept in an incubator with humidified 5% CO_2/95% air at 37°C. In preparation for recording cells were plated at low density on acid-washed glass coverslips cultured for at least one day. No difference in expression levels or properties of the currents were seen when cells were grown in the presence of 5 mM glucose.

Freshly dissociated β-cells were also used in this study. Islets of Langerhans from adult pigs were isolated and purified by collagenase digestion and Ficoll-gradient separation techniques. They were generously provided by the Islet Transplantation Laboratory of Washington University (St. Louis, MO). The islets were dispersed into single cells according to a protocol modified from Lemmark 1974. Briefly, islets were incubated in trypsin-EDTA, washed, reincubated and then triturated with a pipetter using 200 µl siliconized microtips. Subsequently the isolated cells were plated at various densities in 3 ml culture dishes containing sterile glass coverslips. The islet cells attached to the coverslips within 2 hrs and and were recorded from for up to two weeks. The cells were maintained in a 37°C incubator in CMRL medium (Sigma, St. Louis, MO) with 10% FBS, 11.1 mM glucose and penicillin/streptomycin.

Solutions for recording of $I_{K(Ca)}$

In most experiments a standard internal solution was used with K^+ as the main cation. The composition of the solution was (in mM): 50 K_2SO_4, 60 KCl, 1

$MgCl_2$, 10 HEPES, pH=7.2. In some experiments the internal solution also contained 10 mM KF substituted for KCl. Another intracellular solution had the following composition: 25 mM KCl, 125 mM K-Gluconate, 1 mM $MgCl_2$; 10 mM HEPES, pH=7.2. The properties of the currents were indistinguishable with both solutions. The external solutions were: **B-30 K^+** - 30 mM KCl, 10 mM TEA, 2 mM $CaCl_2$, 115 mM N-methyl-D-glucamine, 10 mM HEPES, pH=7.2 w. HCl; **B-100 K^+** - 100 mM KCl, 10 mM TEA, 2 mM $CaCl_2$, 45 mM N-methyl-D-glucamine, 10 mM HEPES, pH=7.2 w. HCl; **B-30 Tl^+** - 30 mM $TlNO_3$, 125 mM $NaNO_3$, 2 mM $CaCl_2$, 10 mM HEPES. pH=7.2 w HCl. In **B-30 Rb^+, B-30 Cs^+, B-30 NH_4^+** solutions, the 30 mM KCl was replaced by equimolar concentration of the chloride salt of the given cation.

In pig islet cells the K(Ca) current amplitude was substantially lower than in βTC-3 cells. In many cells, therefore, the amplitude of the recorded K(Ca) current was increased by increasing $[Ca^{2+}]_o$ from 2 mM to 4 mM. This increase in Ca^{2+} concentration did not have noticeable effects on the delayed rectifier and A-type K^+ current in pig cells.

Solutions for recording the $I_{Cl(Ca)}$

Cesium as the internal monovalent cation was used to reduce the contaminating outward potassium currents. Most experiments were done with the following pipette solution containing 50 mM Cs_2SO_4, 50 mM CsCl,

10 mM CsF, 1 mM $MgCl_2$, 10 mM HEPES, pH=7.2. Several experiments were performed using a pipette solution which contained 80 mM Cs-Acetate, 50 mM CsCl, 10 mM CsF, 1 mM $MgCl_2$, 10 mM HEPES, pH=7.2. The bathing external solution contained 150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM HEPES, pH=7.2. The pH was adjusted with NaOH or N-methyl D-glucamine. When chloride currents were recorded the external potassium was replaced with 5 mM tetraethylammonium chloride (TEA-Cl) to block Maxi-K(Ca) currents. In the experiments where the permeability to different anions was measured, the external 150 mM NaCl was replaced with equimolar concentrations of one of the following salts: NaBr, $NaNO_3$, NaI or Na-Acetate. Cl^- substitution experiments for determination of the reversal potential shift were performed using the following extracellular solution (62 Cl^- solution): 53 mM NaCl, 97 mM Na-Isethionate, 5 mM TEA-Cl, 2 mM $CaCl_2$, 10 mM HEPES, pH=7.2. In the experiments where external Na^+ was reduced, 140 mM NaCl was replaced with equimolar amount of tetramethylammonium chloride (TMA-Cl). In some cells in addition to the chloride current there was a large component of an inwardly rectifying potassium current which was not reduced by calcium removal. In these cells 1 mM CsCl added to the external solution, was sufficient to block the inward rectifier (unpublished observations). In the experiments with strontium, barium and magnesium 2 mM external calcium was equimolarly replaced with the divalent ion in question.

Perforated-patch recording and separation of currents

Recordings were performed using the perforated-patch whole-cell mode (Horn & Marty 1988; Falke, Gillis, Pressel & Misler 1989; Sala, Parsey, Cohen & Matteson 1991; Akaike & Harata 1994). This technique is a modification of the standard whole-cell recording mode in that it allows development of electrical continuity between the recording pipette and the cell interior without the danger of washout of cell constituents. What is more important for our study is that this method allows only the passage of monovalent ions between the pipette and cell interior, thus leaving the Ca^{2+} buffering system intact (Akaike & Harata 1994). This optimized our ability to study Ca^{2+}-dependent currents and their hormonal regulation under physiological Ca^{2+} buffering conditions.

For this mode of recording the patch is filled with a solution containing the antifungal agents nystatin and amphotericin B acting as pore formers. The fungicidal activity of these polyene antibiotics, produced by *Streptomycetes* bacteria, results from their ability to form pores in membranes that are permeable to small solutes (Marty & Finkelstein 1975). The channels formed by nystatin and amphotericin B have the pore size of 0.4 nm and 0.8 nm respectively. From studies in lipid films and molluscan neurons it was found that nystatin and amphotericin induce channels that are selectively permeable to monovalent ions such as K^+, Na^+, Cs^+ with no permeability found for

divalents Mg^{2+}, Ca^{2+}, SO_4^{2+} (Myers & Haydon 1972). Permeability to Cl^- has also been reported but it is smaller than that for cations ($P_{anion}/P_{cation} \approx 0.1$).

With identical concentrations of NaCl on both sides of a thin lipid membrane the current-voltage characteristic for nystatin is linear in the ±75 mV range. Thus there is no voltage dependence. Also the conductance does not change with time. Usually the process of exchange of intracellular Na^+, Cs^+ or K^+ occurs very rapidly. With Cl^- ions, on the other hand, the complete exchange requires up to 10 min. Although this is slow, during long recording the internal Cl^- concentration will be altered. Recently, a new technique was developed for studying the membrane currents in cells under physiological internal Cl^- concentrations. This is accomplished by using gramicidin, instead of amphotericin or nystatin as the perforating agent. Gramicidin is a polypeptide produced by *Bacillus brevis* , it forms pores that are permeable to K^+ and Na^+ but not to Cl^- (Hladky & Haydon 1984). Therefore the internal Cl^- concentration will not be altered by diffusion from the pipette (Akaike 1994; Kyrozis & Reichling 1995). The pores formed by gramicidin are also voltage-independent. We employed this technique to determine the equilibrium potential of Cl^- in βTC-3 cells from the $I_{Cl(Ca)}$ reversal potential.

The recordings were done with amphotericin B , nystatin or gramicidin as membrane perforators. Amphotericin B / nystatin stock was prepared in DMSO (6 mg/ml). Amphotericin was sonicated for several seconds to dissolve it completely, then aliquoted and stored at -20 °C for up to three weeks. Nystatin

was dissolved by vortexing and could be stored for about a week (-20°C) without losing activity. Before the experiment 10 µls of stock was dissolved in 2.5 mls of the internal solution, yielding the final antibiotic concentration of 240 µg/ml. Gramicidin D stock was prepared in methanol (1 mg/100 µl) and stored at +4°C for a week. The gramicidin-methanol solution was added to the pipette internal solution to give a final concentration of 14-30 µg/ml. During the recording the microscope light was routinely switched off to prevent the loss of antibiotic activity. As the pipette was approaching the cell, its resistance was constantly monitored by applying 5 mV voltage steps. When an increase in the resistance was detected, suction was applied and the seal resistance reached the gigaohm range. Seal formation with βTC-3 cells was facilitated by applying 20-30 mV to the pipette (intracellular side negative). After the seal formation the pore former diffuses to the surface of the cell and starts to permeabilize the membrane. In a matter of 5-10 minutes the perforation is fairly advanced to start the actual recording. The extent of the established electrical continuity between the pipette and the cell interior was judged by monitoring the increases in capacitive current size in response to a voltage step.

It was possible to perforate the same cell more than once with different pipettes. After the first recording, the pipette was gently removed from the cell surface. The cell generally did not appear stressed in any way. The pore forming effect of nystatin or amphotericin is reversible. In lacrimal gland cells

(Horn & Marty 1988), the membrane permeability returned to the pretreatment levels in 1 min. A fresh pipette with a different internal solution was then used to make a seal and perforate the same cell. Sometimes the size of the current would be reduced in the second recording, presumably due to shrinkage of the cell.

Micropipettes were made of borosilicate glass (WPI, Sarasota, FL, or Kimax-51, Kimble Glass Inc., Toledo, OH), using a programmable puller (Sutter, Novato, CA). The pipette resistances ranged from 1 to 3 MΩ when filled with the recording solution. The bath was grounded through a 2 M KCl agar bridge. The recording chamber was continuously perfused with a gravity-driven perfusion system. The access resistance drop was monitored during the perforation process and recording was started after values below 15 MΩ were reached. Typical access resistances were 4-15 MΩ and would be stable for up to 1.5 hrs. Series resistance compensation provided by the PULSE software was used for access resistances above 10 MΩ.

Ionic currents were recorded using the EPC-9 patch-clamp amplifier and the PULSE/PULSEFIT (v. 7.6) data acquisition software (Heka Electronik, Lambrecht, Germany). Data were stored on the hard disk of a Macintosh Quadra 950 computer or on Syquest removable cartridges for further analysis using the Igor Pro software (WaveMetrics, Lake Oswego, OR). The sampling rate was 1 kHz for most recordings. At this sampling rate the contamination from inward Na^+ current was minimal, since these currents inactivate with a

time constant τ=2 ms in β-cells (Plant 1988). The protocols used to elicit the K(Ca) current were spaced at 10 s or longer intervals to avoid Ca^{2+} overloading artefacts. All experiments presented were performed at room temperature.

Several recordings of the $I_{K(Ca)}$ current at were performed at ≈34°C. Generally the amplitude of the current was larger than at room temperature (not shown).

The possible offsets during the recording were estimated in the following way. Under the same recording conditions, cells were chosen which displayed a significant inwardly rectifying K^+ current. This current was Ba^{2+} and Cs^+ sensitive but insensitive to 100 μM Cd^{2+} (a concentration sufficient to block the Ca^{2+}-dependent conductances). The reversal potential of the inward rectifying current was assessed at various external potassium concentrations by applying Ba^{2+} or Cs^+. The reversal potential was close to the calculated K^+ equilibrium potential. In some cells the reversal potential was shifted to more positive potentials by up to +10 mV. Since this shift did not occur in all the cells tested, we did not correct our results to account for this offset.

Chapter 3

Characterization of voltage-dependent Ca^{2+} current in βTC-3 and pig islet cells

The Ca^{2+} current in βTC-3 cells

The Ca^{2+}-dependent currents described in this study can be activated by external Ca^{2+} influx. Thus, the voltage-dependent Ca^{2+} currents occurring in βTC-3 cells and pig islet cells were briefly characterized. No published reports have described the Ca^{2+} currents in these two preparations.

In mouse β-cells it has been demonstrated that Ca^{2+} currents flow only through channels with properties resembling those of the L-type or high voltage activated (HVA) (Rorsman & Trube 1986; Plant 1988; Smith et al. 1990). They mediate most of the Ca^{2+} influx during the action potential.

Calcium currents were evoked by depolarizing 10 mV steps from a holding potential of -70 mV (Fig. 2A) in 2 mM external calcium (close to physiological) or 20 mM barium. At this holding potential most sodium currents were inactivated, as previously described (Plant 1988). The current-voltage relation for the calcium channel in the same cell is illustrated in Fig. 2A. The inward current becomes visible around -30 mV and reaches its maximum at 0 mV in 2 mM external Ca^{2+} (trace 1) and +10 mV in 20 mM external Ba^{2+} (trace 2). The currents could be completely and reversibly blocked with bath application of 100 μM Cd^{2+} (trace 3, 4), by substituting the external divalents

Fig. 2. The calcium current in βTC-3 cells. **A**, I-V relations were obtained from responses to depolarizing steps as depicted in the inset. The calcium currents were measured in 2 mM external Ca^{2+} (curve 1), 20 mM Ba^{2+} (curve 2) and were blocked with 100 μM Cd^{2+} (curve 3). The block was reversible (curve 4). **B**, the calcium channels do not significantly inactivate during prolonged depolarization and are permeable to both Sr^{2+} and Ba^{2+}. The external divalent ion concentrations were 2 mM Ca^{2+} (trace 1), 2 mM Sr^{2+} (trace 2) and 20 mM Ba^{2+} (trace 3). Internal solution: B-I-Cs-sulphate, external solution: B-5TEA (0 mM K^{+}).

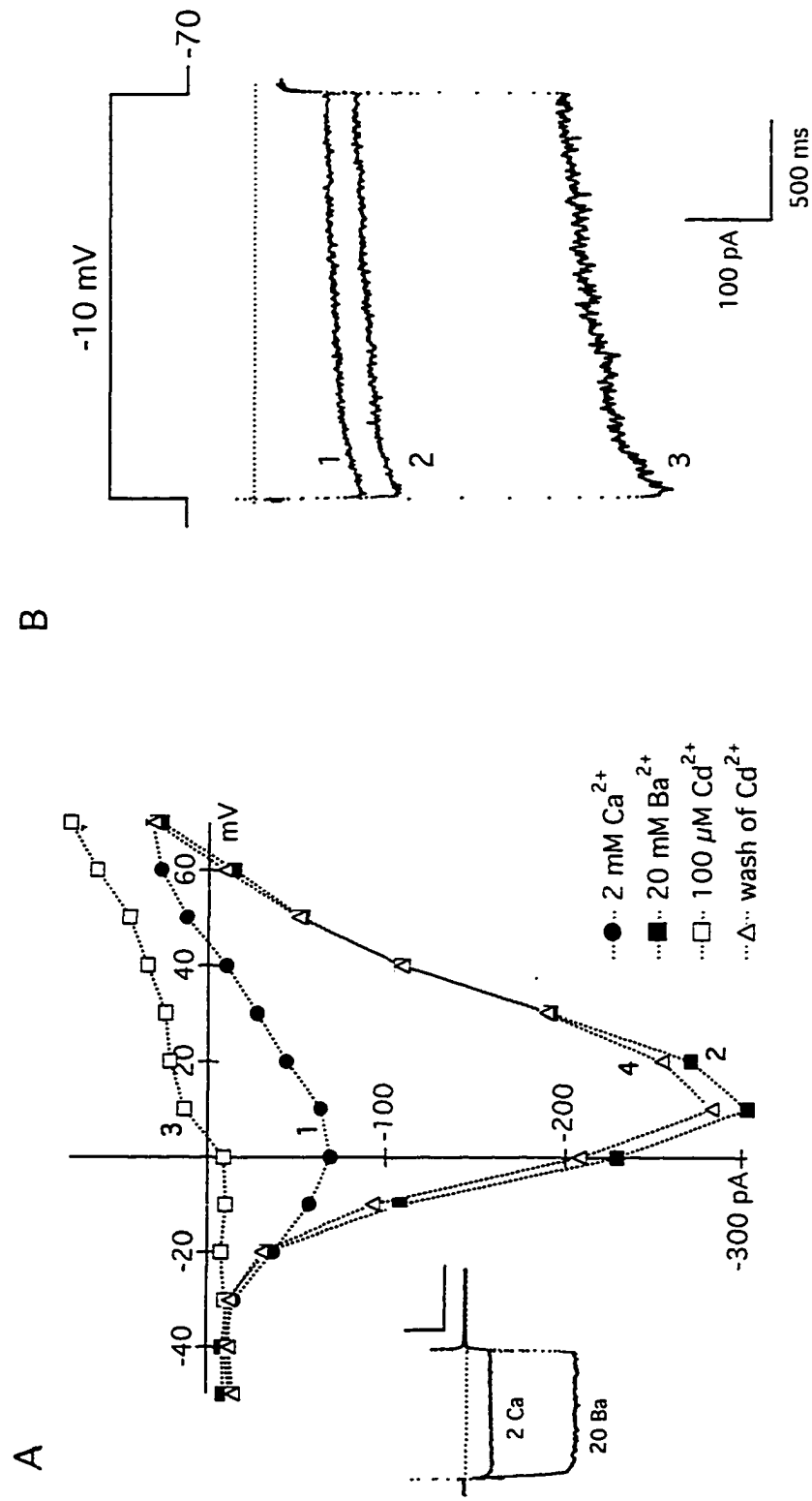


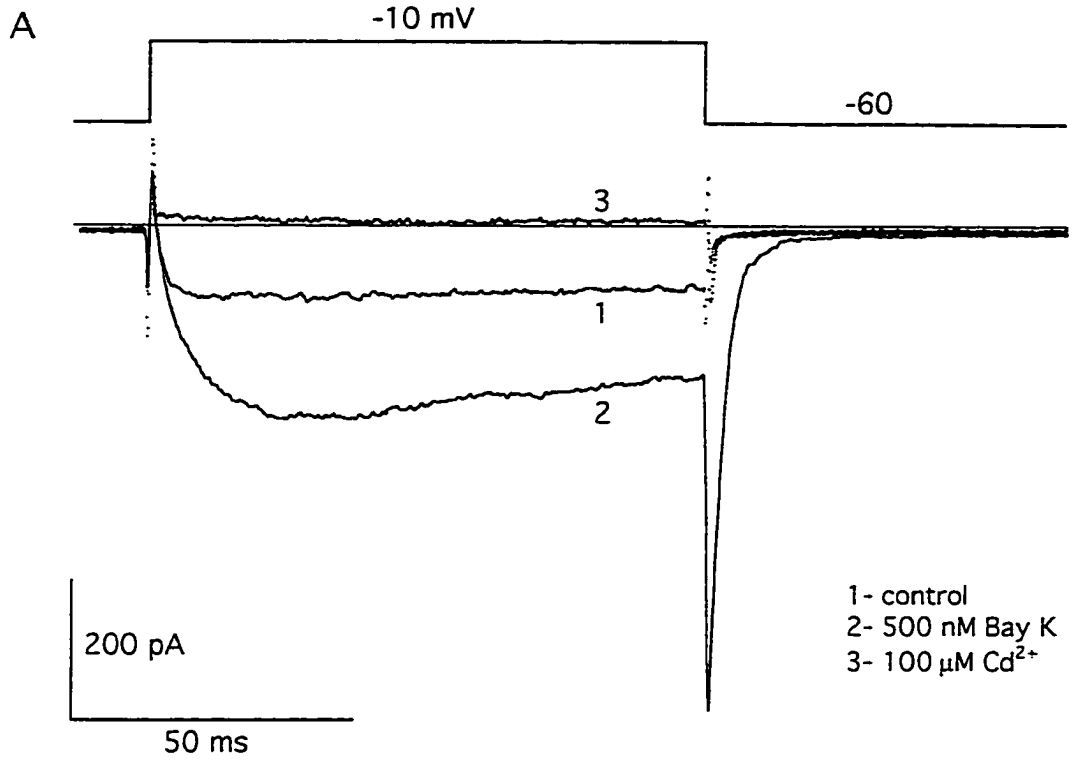
Fig. 2

with Mg^{2+} or simply by omitting them (data not shown). The positive shift in the peak voltage in 20 mM external Ba^{2+} is most probably due to the screening of the membrane surface charges by high divalent ion concentrations (Hille 1992). Sr^{2+} ions were also permeant through the channels as shown in Fig. 2B. The currents showed little inactivation during application of depolarizing steps which were as long as 2 s (Fig. 2B).

Dihydropyridines nifedipine and Bay K 8644 are commonly used to pharmacologically identify L-type, HVA channels (Tsien 1983). in βTC-3 cells nifedipine (100 nM) consistently eliminated the inward Ca^{2+} current (n=4, data not shown). Fig. 3 shows the effect of L-type channel agonist Bay K 8644 on the Ca^{2+} current. In panel A Ba^{2+} current is evoked by depolarizing to -10 mV (trace 1). Bay K (500 nM) causes increase in the amplitude of the current and the slowing of the tail (trace 2). Application of Cd^{2+} in the presence of Bay K abolished the current (trace 3). Panel B shows the effect of Bay K on the I-V relationship of the Ba^{2+} current. The threshold for activation is shifted to a more negative potential value (-40 mV) and so is the peak current (-10 mV). All the characteristics of the Bay K effect (increased amplitude, slowing, shift of peak current and activation threshold) are similar to its effects on L-type channels in other tissues (Hess 1990).

Another common L-type antagonist, verapamil, blocked the βTC-3 Ca^{2+} currents reversibly at 100 μM concentration (n=2).

Fig. 3. Dihydropyridine Bay K 8644 potentiates the voltage-dependent Ca^{2+} current in βTC-3 cells. **A.** 100 ms long voltage steps were applied from holding potential of -60 mV in the absence (1) and presence (2) of 500 nM Bay K. Cd^{2+} blocked the current entirely. Ba^{+} (2 mM) was the charge carrier to avoid activation of the Ca^{2+}-dependent currents. Note the slowdon of the Ba^{2+} tail current in Bay K. **B.** A current-voltage relationship generated from reponses shown in **A** (same cell). The peak Ba current in the presence of Bay K is shifted to a more negative value. Internal solution: B-I-K-sulphate, external solution: B-5K, 2 mM Ba^{2+} , 10 mM TEA.



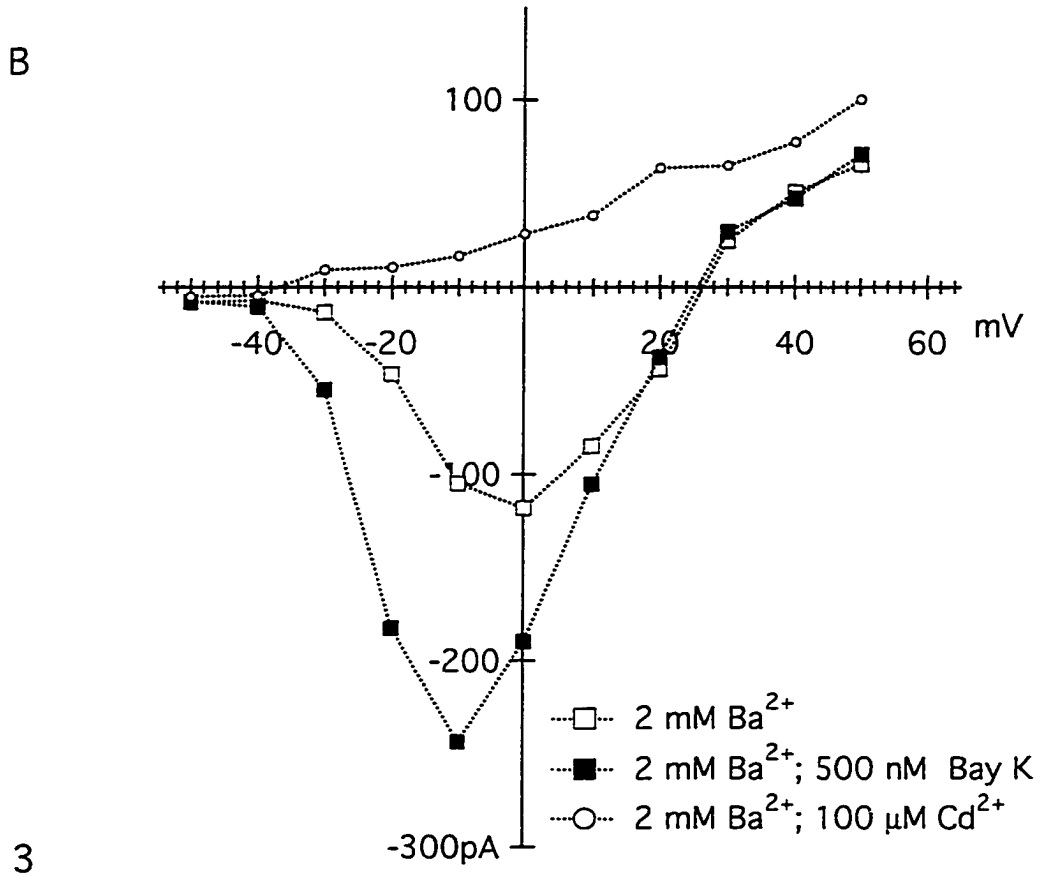


Fig. 3

In the absence of external divalent cations the Ca^{2+} channel was permeable to monovalents (in our case Na^+). Fig. 4 shows a family of Na^+ currents through Ca^{2+} channels evoked by the same protocol as in Fig. 2A, when external solution contained 0 added Ca^{2+} and 1 mM EGTA (0 Ca^{2+}-EGTA solution) to chelate the contaminating divalents. Panel B depicts the current-voltage relationship generated from the data shown in A. The peak voltage is shifted towards more negative voltages (-10 mV) and the threshold depolarization is also shifted in the same direction (-40 mV). Panel C shows a comparison of the I-V relation from a voltage ramp in the presence of external Ca^{2+} (trace 1) and in the absence of divalents (trace 2). A reduction of outward current ($I_{K(Ca)}$ -see chapter 5) is apparent. The remaining outward current seen in the 0 Ca^{2+}-EGTA solution is mostly the current carried through the Ca^{2+} channel by K^+, the main intracellular cation. Ramps were used routinely to screen the cells for expression of particular currents (e.g. $I_{Cl(Ca)}$, $I_{K(Ca)}$, I_K-IR, I_K-DR etc). The current activated in response to a longer depolarizing step is shown in panel D. It can be seen that the inactivation properties of the current carried by Na^+ do not differ greatly from the one shown in Fig. 2B with Ba^{2+} or Ca^{2+} as the charge carrier. The current (carried by Na^+) is not diminished greatly at the end of the 6 s step. The movement of monovalent cations through the L-type (and other types, such as T-type channels) channels is commonly observed in other preparations in the absence of external divalents (Tsien, Hess, McCleskey & Rosenberg 1987). The crossover point in Fig. 4B is more positive (close to 12 mV) than with Ca^{2+} as the charge carrier. It is important to

Fig. 4. The Ca^{2+} current in βTC-3 cells is permeable to Na^{+} in the absence of external divalents. **A.** Current through Ca^{2+} channels carried by Na^{+}. Standard depolarizing voltage steps were applied (100 ms duration) in an external solution containing 5 mM K^{+}, 140 mM Na^{+} and 1 mM EGTA. **B.** I-V plot obtained from the reponses in panel A. A shift in the peak current towards a more negative value is seen (-10 mV). **C.** The I-V generated from a voltage ramp protocol in the presence of external 2 mM Ca^{2+} (1) and in the absence of divalents (2). **D.** The current carried by Na^{+} elicited by a long depolarizing step to 0 mV (6 s duration). The holding potential was -60 mV. The internal solution was: B-I-K-sulphate.

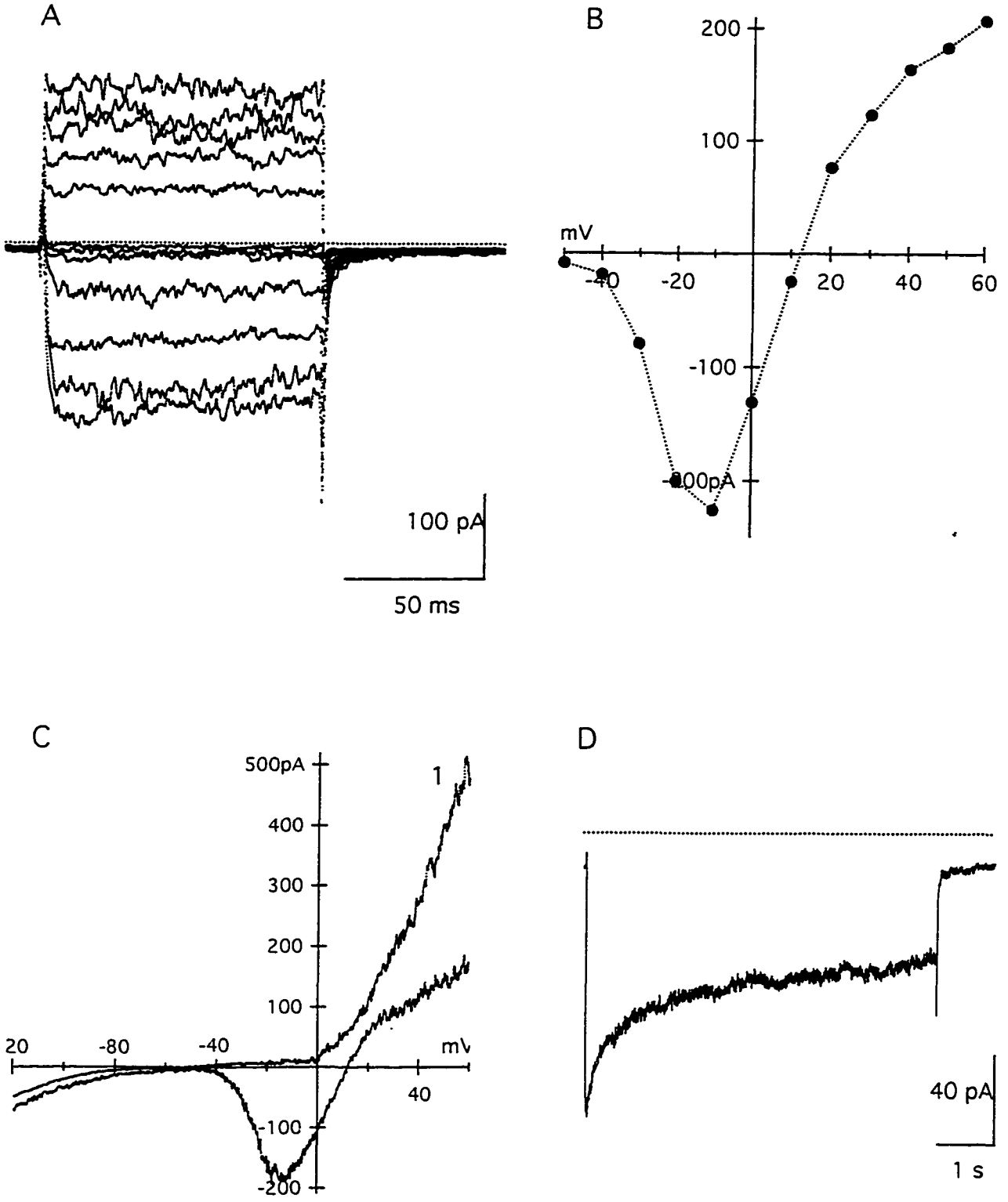


Fig. 4

note that this is not a true reversal potential of the current. The exact reversal potential could not be determined because Cd^{2+} failed to block the current through the Ca^{2+} channels in the absence of divalents. Most likely the outward current through the Ca^{2+} channels is carried by K^+ ions.

No rapidly inactivating (T-type) currents were observed when the cell membrane was depolarized from more negative holding potentials potentials (-90 mV).

Based on this evidence we conclude that the voltage-dependent Ca^{2+} current in βTC-3 cells is L-type.

The Ca^{2+} current in pig islet cells

The voltage-dependent Ca^{2+} current in pig islet cells displayed slight differences from its counterpart in βTC-3 cells. Fig. 5A depicts a typical Ca^{2+} current (charge carrier is Ba^{2+}) evoked by a standard step to -10 mV, the I-V relation generated from these responses is shown in panel B. It is apparent that the threshold for activation for the I_{Ca} in pig cells is more negative (-40 mV) and it peaks at -10 to 0 mV (n=7), negatively shifted from that in βTC-3 cells. Fig. 5C shows the Ba^{2+} current evoked by a long (6 s) duration depolarizing

Fig. 5. The calcium current in pig islet cells. **A.** 100 ms step to -10 mV elicits an inward current carried by Ba^{2+} (2 mM) blocked by 100 μM Cd^{2+}. The cadmium effect was reversible (not shown). **B.** The I-V plot generated from traces like in A in the presence and absence of Cd^{2+}. **C.** The barium current elicited by a long depolarizing step to -10 mV (6 s duration). Note the inital inactivating phase. The holding potential was -80 mV. The internal solution: B-I-K-Sulphate, external solution: B-5K, 2 mM Ba^{2+}. This cell had a large Na^{+} current, therefore 2 μM TTX was applied throughout the experiment.

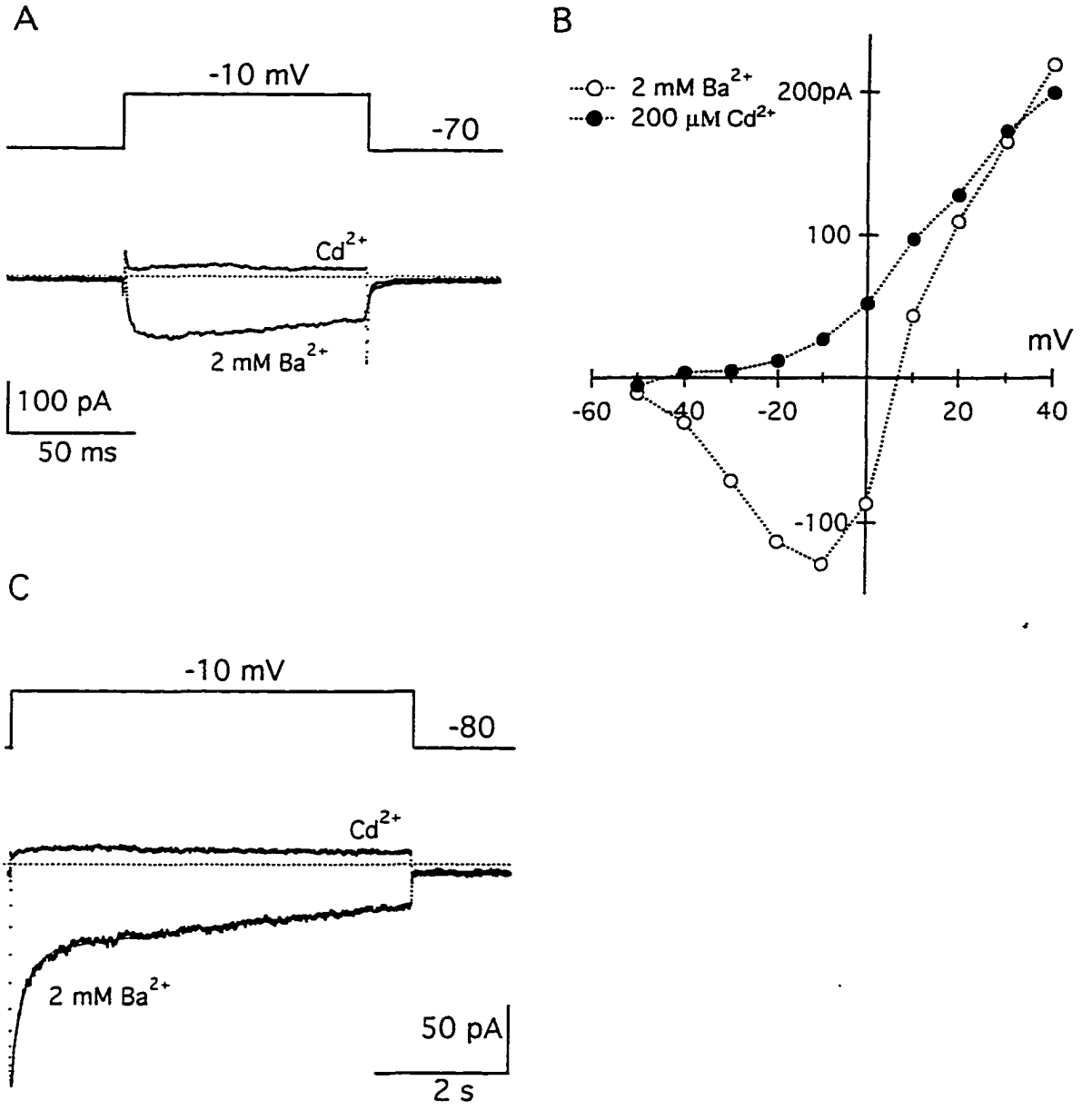


Fig. 5

step. The current consists of two components- fast inactivating and sustained. The presence of the fast inactivating initial component differentiates it from the I_{Ca} in βTC-3 cells. Whether this inactivating component constitutes a T-type current was not clarified in this study. Usually the T-type channels activate at more negative potentials (below -50 mV) and can only be evoked from very negative holding potentials (-90 to -100 mV) (Hess 1990). We did not notice marked reduction of the inactivating portion of I_{Ca} at more postive holding potentials (-60 mV), which makes it unlikely that T-type channel is present in these cells. Also, the time constant of the fast component was about ≈200 ms, much slower than for the described T-type channels.The paucity of the data from pig islet cells does not allow us to rule out the presence of Ca^{2+} channels other than L-like.

Chapter 4

The Ca^{2+}-dependent Cl^- current

A depolarization-induced current requires influx of calcium

Depolarizing voltage steps applied to the membrane in 2 mM external calcium reproducibly activated outward currents followed by slow inward tail currents upon membrane repolarization (Fig. 6A, panel 1). Both outward and inward tail currents were abolished when 100 μM Cd^{2+} was added externally (panel 2), in a reversible manner (panel 3). Fig. 6B illustrates the "onset" and tail I-V relations derived from the same recording as in panel A, in the presence or absence of external Cd^{2+}. The onset peak current and the inward tail current (15 ms after the start of the hyperpolarizing voltage jump) were measured and plotted against the voltage of the depolarizing step. In the absence of cadmium, the I-V relation of the outward current is bell-shaped with a maximum at +20 mV. In cadmium the I-V collapses into the voltage axis since currents at all voltages are blocked. It is apparent that the tail current I-V relation resembles that of the Ca^{2+} current depicted in Fig. 2B. Both are bell-shaped and parallel. The threshold voltages for activation of the tail and calcium currents are also similar. The tail currents (as well as the outward currents) are diminished at voltages higher than +50 mV, close to the apparent calcium reversal potential. The concentration of cadmium used is sufficient to block the calcium current present in the cell, as discussed in Chapter 3. As seen in Fig. 6C, an L-type Ca^{2+} channel blocker verapamil (200 μM)

Fig. 6. Peak and tail current-voltage relationships of the depolarization-induced current. **A**, depolarizing pulses evoked slow outward current followed by a slow inward current upon repolarization to the holding potential (panel 1). 100 μM Cd^{2+} blocked completely the evoked currents (panel 2). The Cd^{2+} effect was reversible upon its removal (panel 3). **B**, I-V curve was obtained from the raw traces shown in A. The peak outward and inward (tail) currents were measured (at the arrows in **A**1 and **A**2) and plotted against the voltage at which they were evoked. The internal solution was B-I-Cs-sulphate, external solution: B-5TEA, 0 mM K^+. **C**. L-type Ca^{2+} channel blocker verapamil (200 μM) reversibly abolished the depolarization-induced currents.

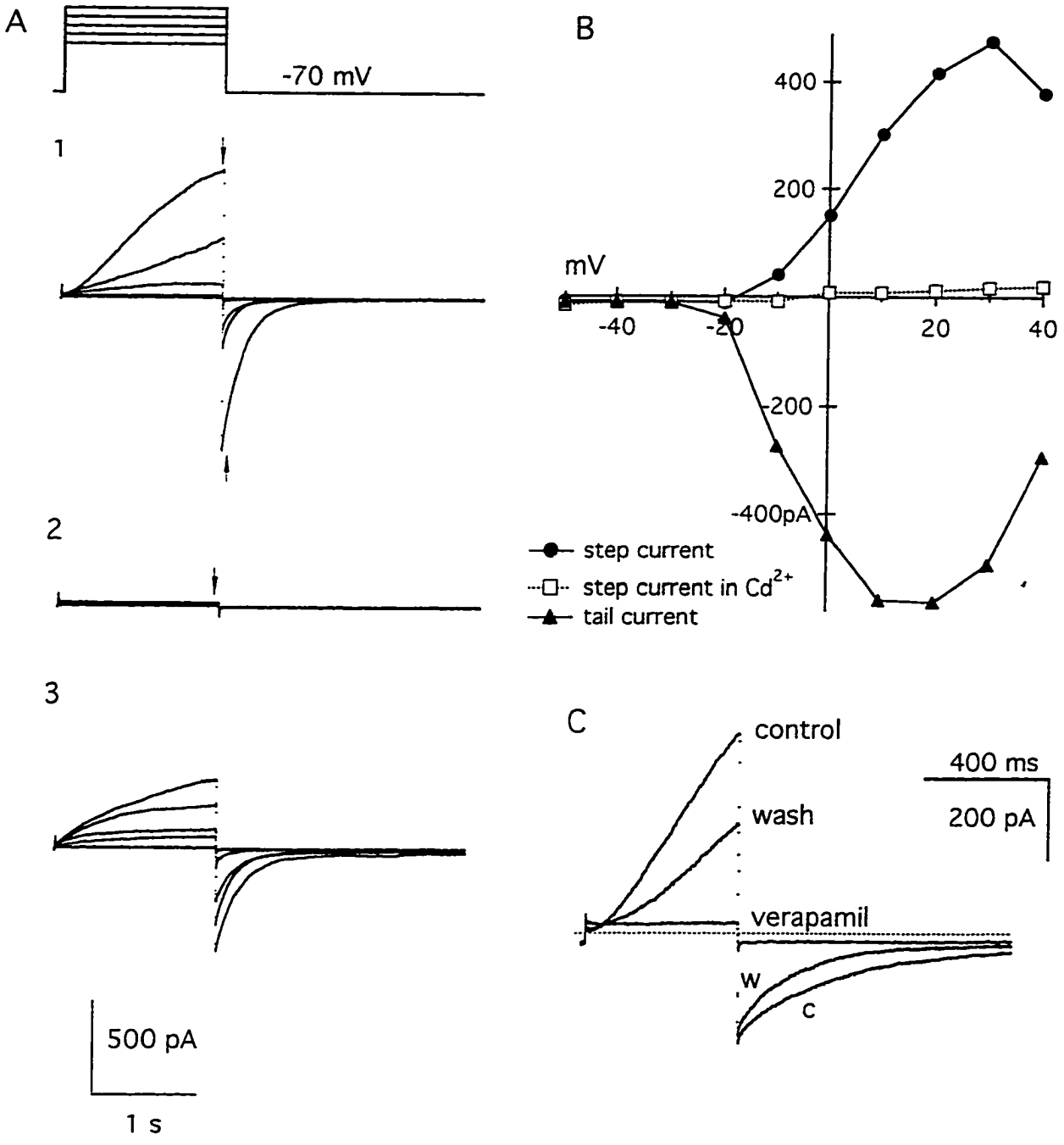


Fig. 6

also abolishes the depolarization-induced currents. Collectively these observations suggest that the voltage-induced outward currents and the corrseponding tails require Ca^{2+} influx through the voltage-gated Ca^{2+} channels (L-type). One interesting difference between the Ca^{2+}-activated current and I_{Ca} is in their "apparent" voltage dependence. The Ca^{2+}-activated I/V relationship is positively shifted in relation to that of the Ca^{2+}-channel. This may reflect voltage-dependent regulation of this current in addition to its Ca^{2+} dependence (see below).

Replacing the external calcium with magnesium or simply omitting it from the bathing solution had the same effect as cadmium addition (data not shown). All of these observations suggest therefore that the voltage-induced outward currents and the corresponding tails require calcium influx through the voltage-gated calcium channels.

The Ca^{2+}-dependent current increases with duration of the depolarizing pulse

The experiment described in this section demonstrates that the calcium-activated current depended on the duration of the depolarizing pulse and establishes a correlation between the outward current activated by depolarizing voltage steps and the slow tail currents recorded upon repolarization to -70 mV.

Fig. 7A shows outward currents recorded after depolarizations to -10 mV of increasing duration and corresponding tail currents upon repolarization to -70 mV. Both outward and corresponding inward tail currents increased with the duration of the pulse. We plotted the peak outward current amplitude against the amplitude of the inward tail current 15 ms following the corresponding voltage step. The tail current amplitude correlated closely with the amplitude of the "onset" outward current (Fig. 7B), suggesting that both currents flow through the same channels. This result is consistent with a progressively greater calcium influx resulting from the increased duration of the depolarizing pulse (see Fig. 2B and also Rorsman & Trube 1986), which in turn activates a larger Ca^{2+}-dependent conductance.

The Ca^{2+} effect cannot be mimicked by Ba^{2+} ions

The HVA Ca^{2+} channels in βTC-3 cells show permeation to Ba^{2+} and strontium ions as well (see Fig. 2). These divalent ions were tested to determine whether they could substitute for calcium in activating the Ca^{2+}-dependent current. Fig. 8 shows the outward current induced by a depolarizing step to +10 mV along with the corresponding tail current in normal (2 mM) external Ca^{2+} (trace 1). Replacement of Ca^{2+} with equimollar strontium, reduced the Ca^{2+}-dependent current after a delay (n=5). Subsequent addition of 100 μM Cd^{2+} did not block the remaining current (not shown). In some cases strontium substitution abolished the Ca^{2+}-dependent

Fig. 7. The effect of increasingly longer depolarizing pulses on the outward and inward currents ("envelope" test). **A**, depolarizing pulses of increasing durations were applied from a holding potential of -70 mV and tail currents recorded upon repolarization to the holding potential. **B**, the peak outward current for a given step (panel A) was plotted against the corresponding peak tail current to examine the correlation between the two values. Internal solution: B-I-Cs-sulphate, external solution: B-5TEA, 0 mM K^+.

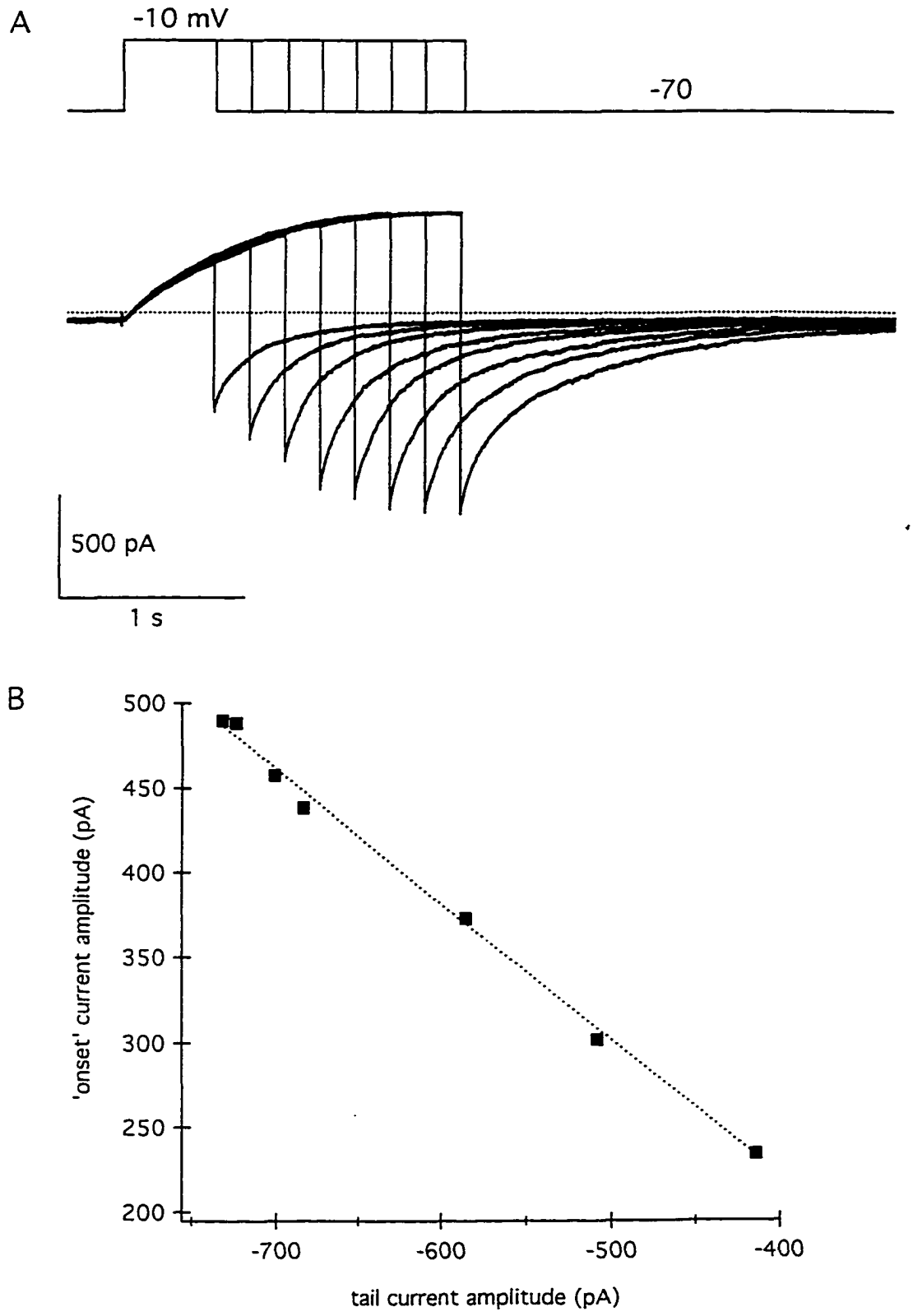


Fig. 7

current (n=4). The effect of Ba^{2+} substitution is shown: the Ca^{2+}-dependent current was abolished (n=5). The Ca^{2+} current in this particular cell at concentrations higher than 2 mM.

Deactivation kinetics

We investigated the voltage dependence of the tail deactivation kinetics. Tail currents were well fitted by a single exponential function. Fig. 9A shows the dependence of the tail current decay time constant, τ (obtained from repolarizations to -70 mV), on depolarizing steps in the voltage range where I_{Ca} is activated. The deactivation time constant shows a bell-shaped dependence on depolarizing step voltages, just as we obtained earlier with the peak tail current (see Fig. 6B). This result suggests that the Ca^{2+}-activated current decays with a time course that depends on the speed of Ca^{2+} buffering in the cytoplasm. The deactivation time constants are similar to the fluorometrically determined constants of the $[Ca^{2+}]_i$ transients caused by L-type channel activation in mouse β-cells (≈1.4 s) (Rorsman et al. 1992). Although this bell-shaped pattern of the deactivation rate dependence on voltage was highly reproducible, the absolute values of the tail current decay times varied widely from one cell to another. Reasons for this large disparity may include differences in initial calcium concentration and rates of calcium sequestration in different cell was relatively large, which allowed us to see the underlying

Fig. 8. Sr^{2+} but not Ba^{2+} can substitute for Ca^{2+} in activating the Ca^{2+}-dependent current. The control trace (2 Ca) is evoked by stepping the potential to 10 mV. Equimolar substitution of external Ca^{2+} with Sr^{2+} reduced both the outward and the inward (tail) currents. Ba^{2+} substitution abolished both the outward and the tail currents, revealing the underlying I_{ca}. The internal solution was B-I-Cs-sulphate, external solution: B-5TEA, 0 K^+ 2 mM divalents.

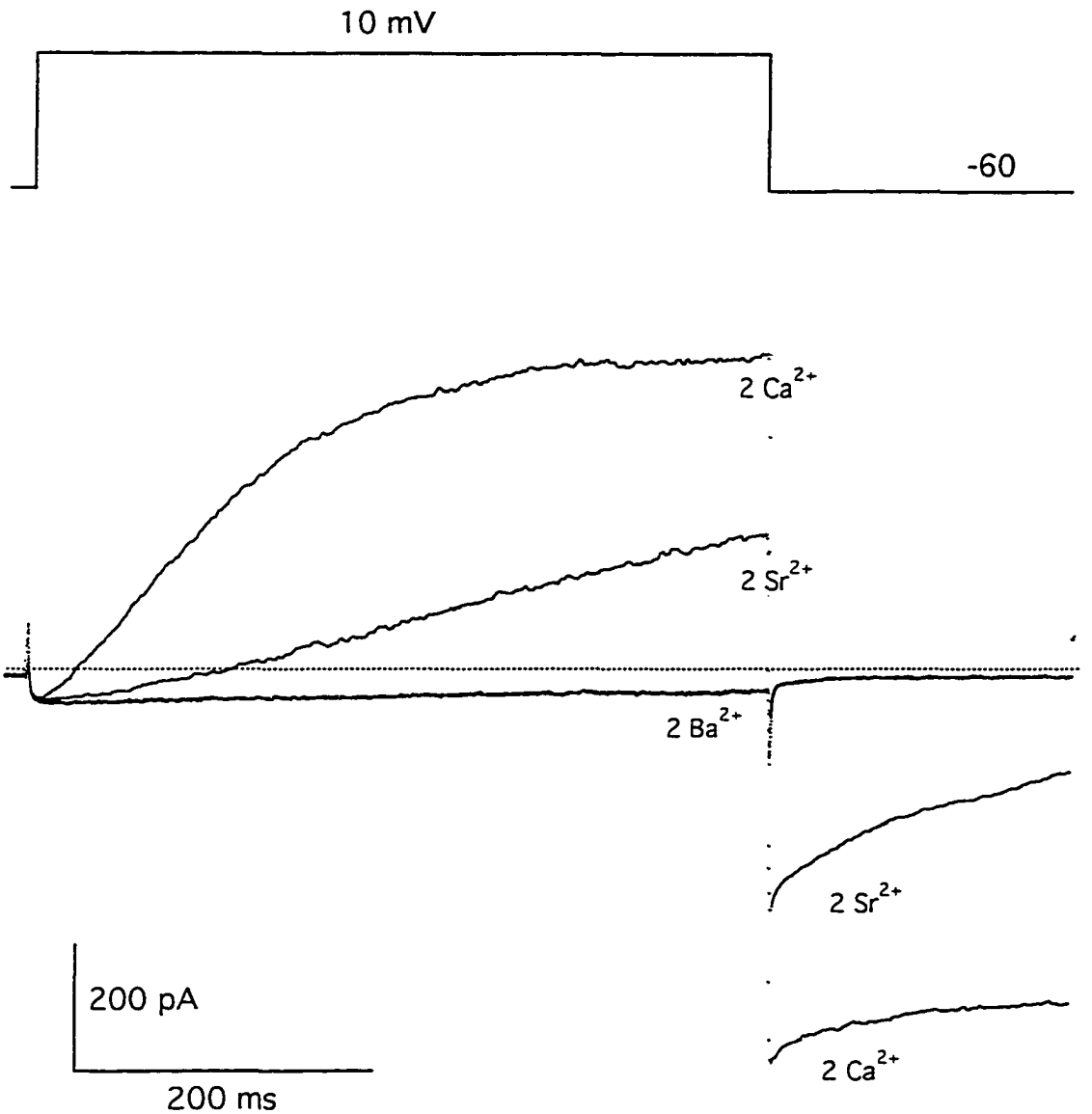


Fig. 8

Fig. 9. Decay kinetics of the tail current dependence on the voltage. In **A**1, the tail currents evoked by various "onset" steps were recorded at the same potential (-70 mV). The tails were fitted with one exponential and the decay time constants (τ) were plotted against the "onset" voltage (**A**2). In **B**1, all the tail currents were generated by a step to +10 mV and recorded at different potentials (-100 mV to -40 mV). The decay time constants form monoexponential fits were plotted against the tail voltage (**B**2). Internal solution: B-I-Cs-sulphate, external solution: B-5TEA.

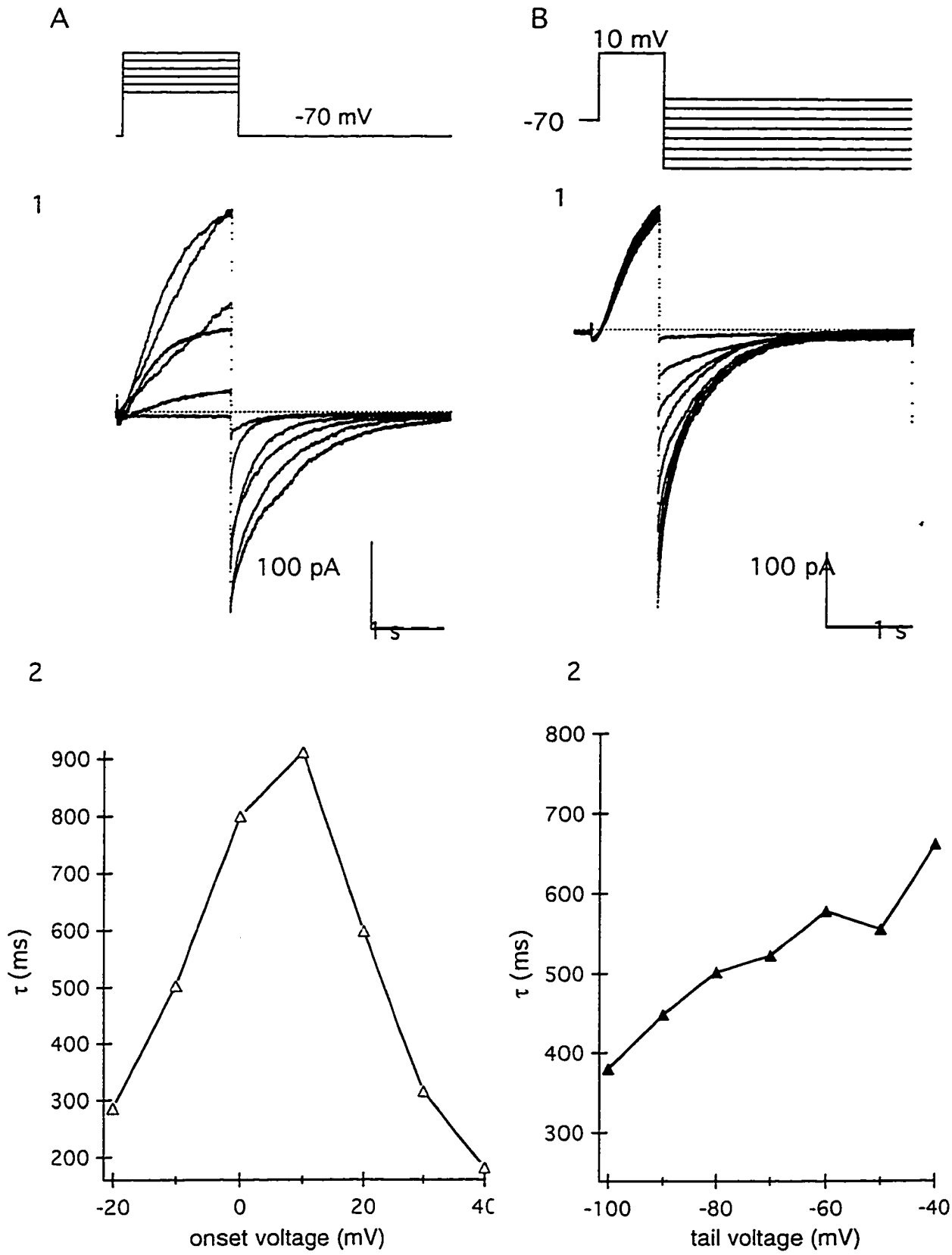


Fig. 9

inward current (carried by Ba^{2+}). We did not study Ca^{2+} substitution by strontium or Ba^{2+} ions.

We also measured the voltage-dependence of decay rates of tail currents within a voltage range where no additional Ca^{2+} channel activation occurred. Fig. 9B shows the protocol involving repolarizations from the same depolarizing step to varying tail potentials (-100 to -40 mV) and plotting of each decay time constant (τ) as a function of the voltage at which the tail current was recorded. In agreement with other studies (Mayer 1985; Korn & Weight 1987; Lamb, Volk & Shibata 1994) we observe that the decay rate is faster the more negative the tail potential is.

The reversal potential of the Ca^{2+}-dependent current and its dependence on extracellular Cl^-

We proceeded to measure the reversal potential of the Ca^{2+}-dependent current as shown in Fig. 10A. A constant depolarizing (conditioning) step was given, followed by repolarizing voltage jumps to a series of potentials, where the tail currents were measured in the presence (panel 1) or absence of extracellular Ca^{2+} (panel 2). The amplitude of each tail current was measured 15 ms following the repolarization step and plotted against the voltage of the step (Fig. 10B). The reversal potential of the Ca^{2+}-sensitive current was extrapolated from the cross-over point. The mean reversal potential estimated by this method was -20.6 ± 2.1 mV (n=12, mean ± SEM), a value close to the

Fig. 10. The reversal potential of the calcium-dependent tail current is consistent with a chloride current. **A**, tail currents evoked by a step to +10 mV in 2 mM Ca^{2+} (1) and in 0 mM Ca^{2+} (2). **B**, the peak tail current-voltage relation in normal external (159 mM) and low (62 mM) Cl^- concentration. The reversal potential in the lower chloride concentration could not be directly determined from tail currents and was extrapolated. Internal solution: B-I-Cs-sulphate, external solution: B-5TEA, 97 mM Na-Isethionate.

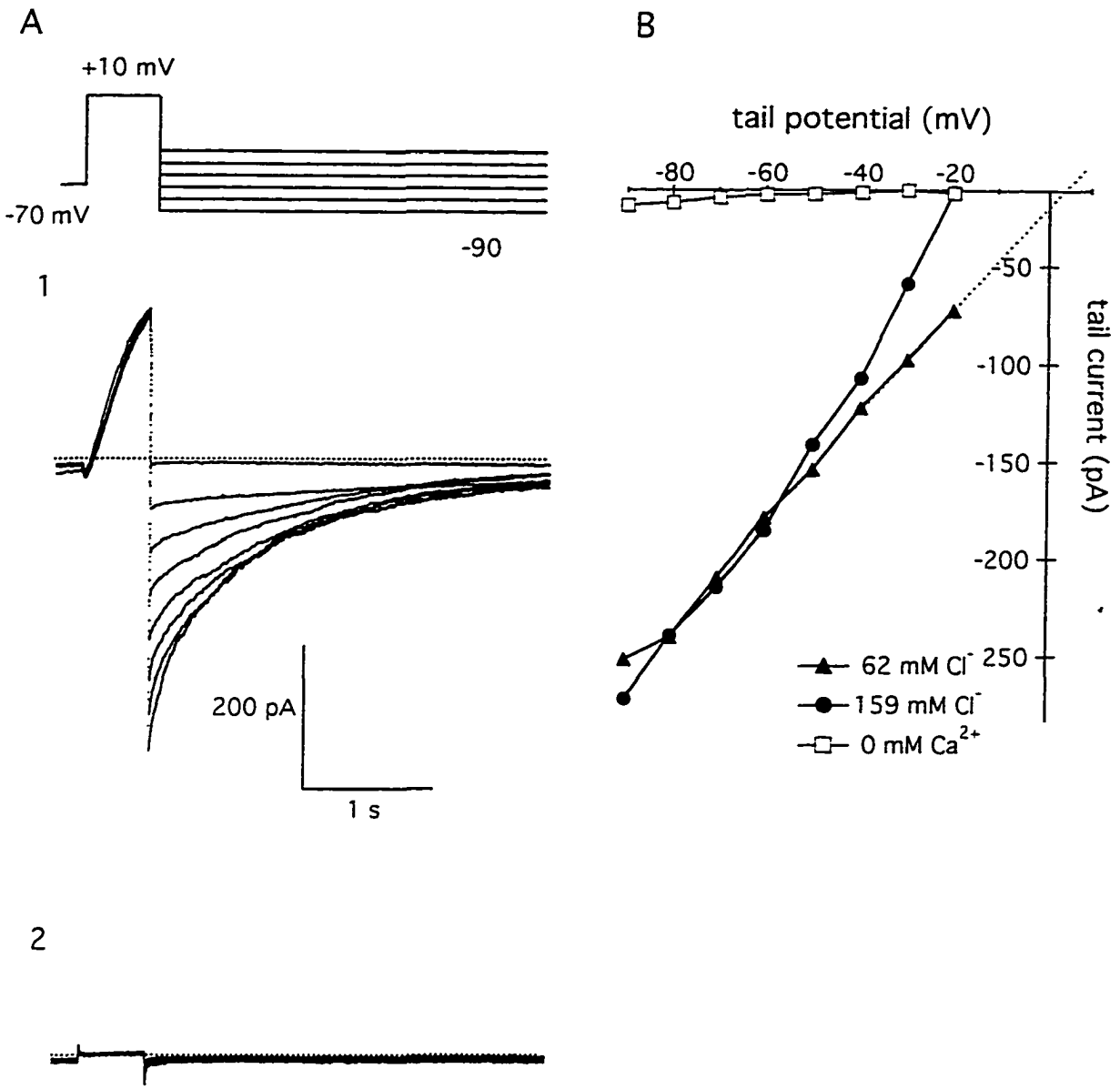


Fig.10

calculated Nernst chloride equilibrium potential of -23.7 mV. The external chloride concentration was further reduced from 159 mM to 62 mM, substituting it with isethionate, an impermeant ion (Franciolini & Petris 1990). As seen on Fig. 10B, the estimated reversal potential shifted by approximately +22 mV, in agreement with the Nernst calculated shift (+23.7 mV). Since the tail current amplitude was measured after a 15 ms delay, it is unlikely that it was contaminated by the "L-like" calcium tail current, which is considerably more rapid (e.g. Plummer, Logothetis & Hess 1989).

The reversal potential changes were also determined from the measurements of the "onset" current amplitude at different depolarizing voltage steps. In Fig. 11 a family of depolarization-induced currents in a βTC-3 cell is shown, in bathing solutions containing 159 mM (11A) or 62 mM (11B) external chloride concentrations. Fig. 11C shows the I-V relations, obtained with these two different external chloride concentrations. The average shift was 20±1.08 mV and the average reversal potential in 62 mM Cl^- was +1.5 mV (n=4). Thus, both methods of reversal potential estimation gave similar results consistent with the assertion that both "onset" and tail Ca^{2+}-dependent currents represent the same Cl^--selective conductance.

The reversal potential of the Ca^{2+}-dependent Cl^- current measured using the gramicidin-perforated patch recording

Fig. 11. The reversal potential of the "onset" currents depends on the extracellular Cl^- concentration. **A**, **B** the Ca^{2+}-dependent currents evoked by depolarizations in 159 mM (**A**) and 62 mM (**B**) external Cl^- concentrations. **C** shows the "onset" I-V relation obtained from the traces in **A** and **B**. The reversal potential can be directly measured in 62 mM Cl^-. Internal solution: B-I-Cs-sulphate, external solution: B-5TEA, 97 mM Na-Isethionate.

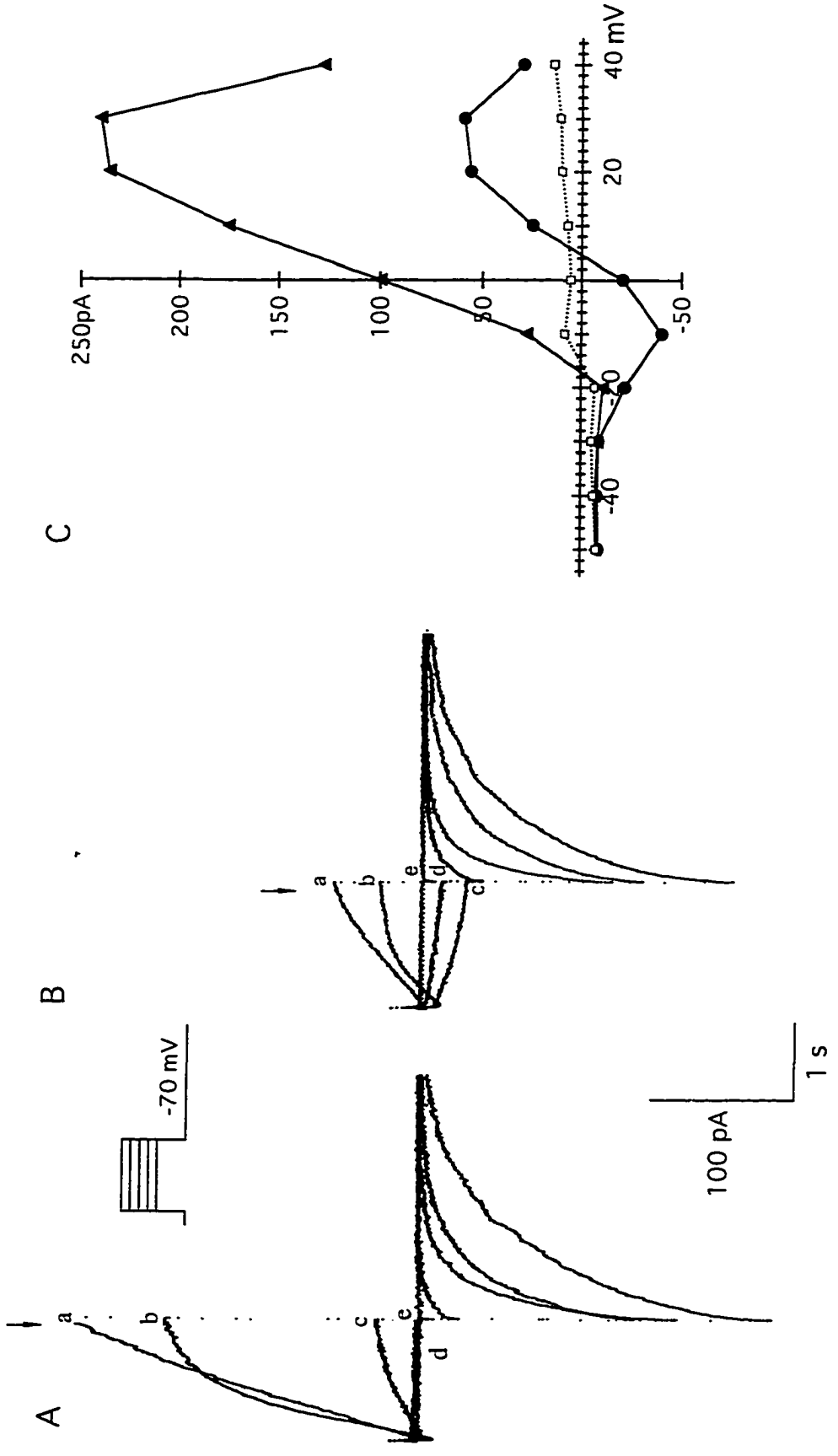


Fig. 11

As described in *Methods* section, gramicidin has been used recently as the perforating antibiotic for studying Cl^- currents, since the channels formed by gramicidin are permeable to cations only and do not alter the intracellular [Cl^-]. In order to estimate the physiological inracellular Cl^- ion concentration in βTC-3 cells, we therefore measured the reversal potential of the $I_{Cl(Ca)}$ using gramicidin in the pipette. The paradigm was the same as in Fig. 10. The reversal potentials were determined at two pipette Cl^- concentrations: 22 and 142 mM, giving similar values which were therefore lumped together. The reversal potential was -22.20±0.93 mV (n=5, mean±SEM). This corresponds to approximately 65 mM internal [Cl^-].

Effects of Cl^- channel blockers on the Ca^{2+}-dependent current

Both the inward tail and the outward Ca^{2+}-dependent currents were blocked by niflumic acid (n=4), a chloride channel blocker, which has been shown to block calcium-dependent chloride channels in other tissues (Franciolini & Petris 1990). In Fig. 12A $I_{Cl(Ca)}$ was evoked by depolarizing voltage steps to 0 and +20 mV in the absence (trace 1) and the presence (trace 2) of 100 μM niflumic acid. Niflumic acid effect could be completely reversed (trace 3). The dose response relationship of the niflumic acid block at various potentials (in another cell) is depicted in Fig. 12B. DIDS also could

Fig. 12. Niflumic acid blocks the Ca^{2+}-dependent Cl^- current. **A.** trace 1, control Ca-dependent current evoked by steps to -20, 0 and 20 mV, **A**, trace 2, block by 100 μM niflumic acid, **A** trace 3, washout of niflumic acid. **B.** Dose response for niflumic acid. The increasing concentrations of niflumic acid (1 μM, 10 μM and 100 μM) were tested on the "onset" current at various potentials (-20 to 40 mV). Internal solution: B-I-Cs-sulphate, external solution: B-5TEA, 150 NaCl.

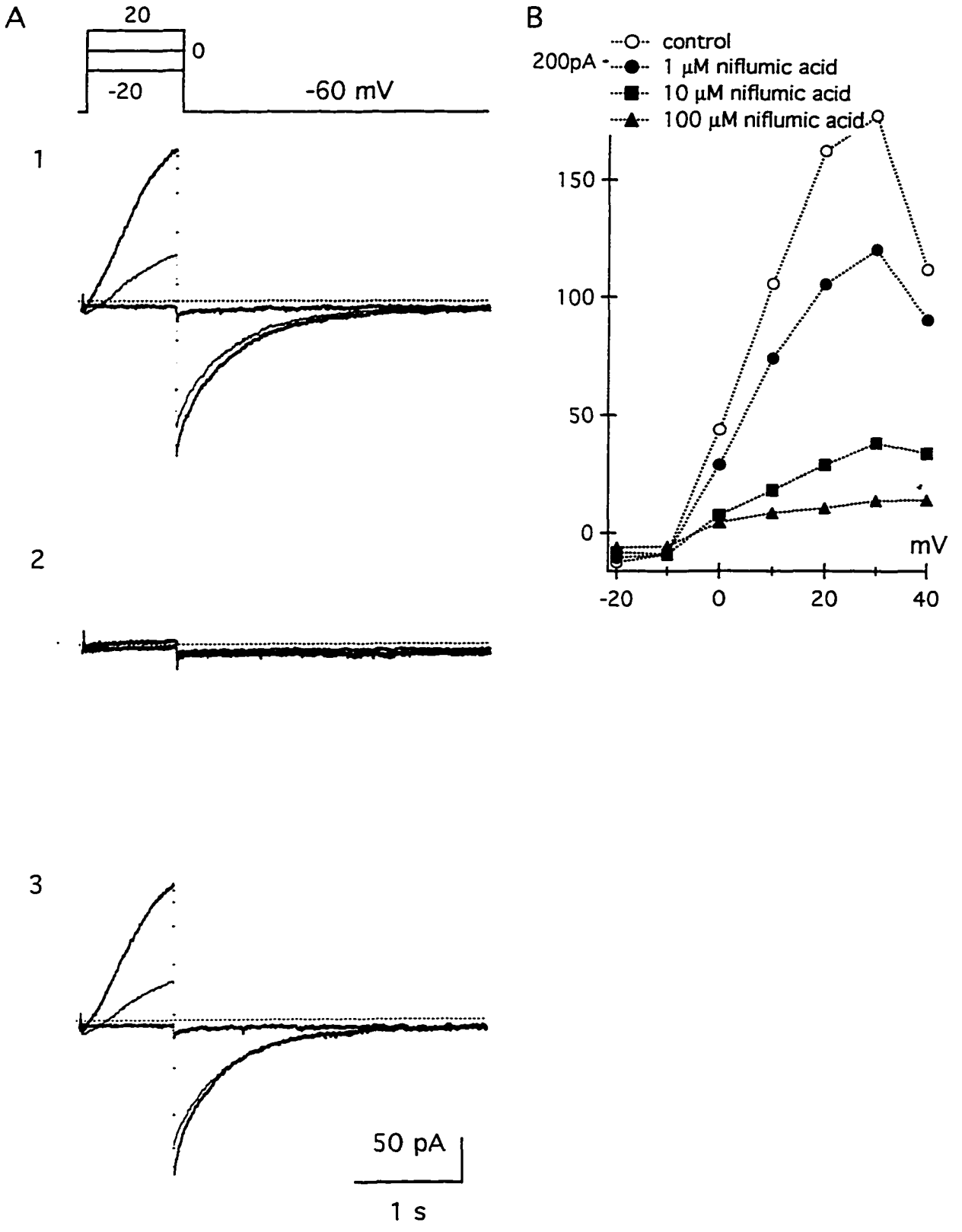


Fig.12

block $I_{Cl(Ca)}$. 400 μM concentration was needed to block approximately 50% of the current (n=5). At this concentration it showed no effects on Ca^{2+} currents (data not shown). Since the Ca^{2+}-activated currents were chloride selective and blocked by chloride channel blockers it was concluded that they were $I_{Cl(Ca)}$.

The tail current was slowed down in the presence of even low conentrations of niflumic acid- 5 μM. DIDS on the other hand, did not affect the channel kinetics.

In addition to DIDS and niflumic acid, 9-AC was tested for block of the $I_{Cl(Ca)}$. In several cells (n=3) 20 μM 9-AC blocked a portion of the current.

Permeability to monovalent anions

In order to determine the ionic selectivity of the $I_{Cl(Ca)}$ 150 mM external Cl^- was replaced with the anion being tested. This anionic substitution left 9 mM Cl^- in the bath. Four monovalent anions were tested and their permeability compared to that of chloride. Fig. 13A shows the depolarization evoked currents when the main external anion is Cl^-, Br^-, NO_3^-, acetate$^-$ or I^-. The current carried by iodide, nitrate and bromide was larger than that carried by Cl^-. Acetate is less permeant than chloride. In order to determine quantitatively the permeability sequence for anions the reversal potentials determined in presence of different anions were measured and the permeability of the given

Fig. 13. The selectivity of the $I_{Cl(Ca)}$ to monovalent anions. **A**, $I_{Cl(Ca)}$ evoked when the main external anion was NO_3^-, Cl^- or I^-. **B**, Determination of the reversal potential of the $I_{Cl(Ca)}$ with Cl^-, Br^- and I^- as the main external anions. Not all traces are shown. **C** shows tail current-voltage relations obtained from the same cell as in **B** for different external monovalent anions (Cl^-, NO_3^-, Br^-, I^-).

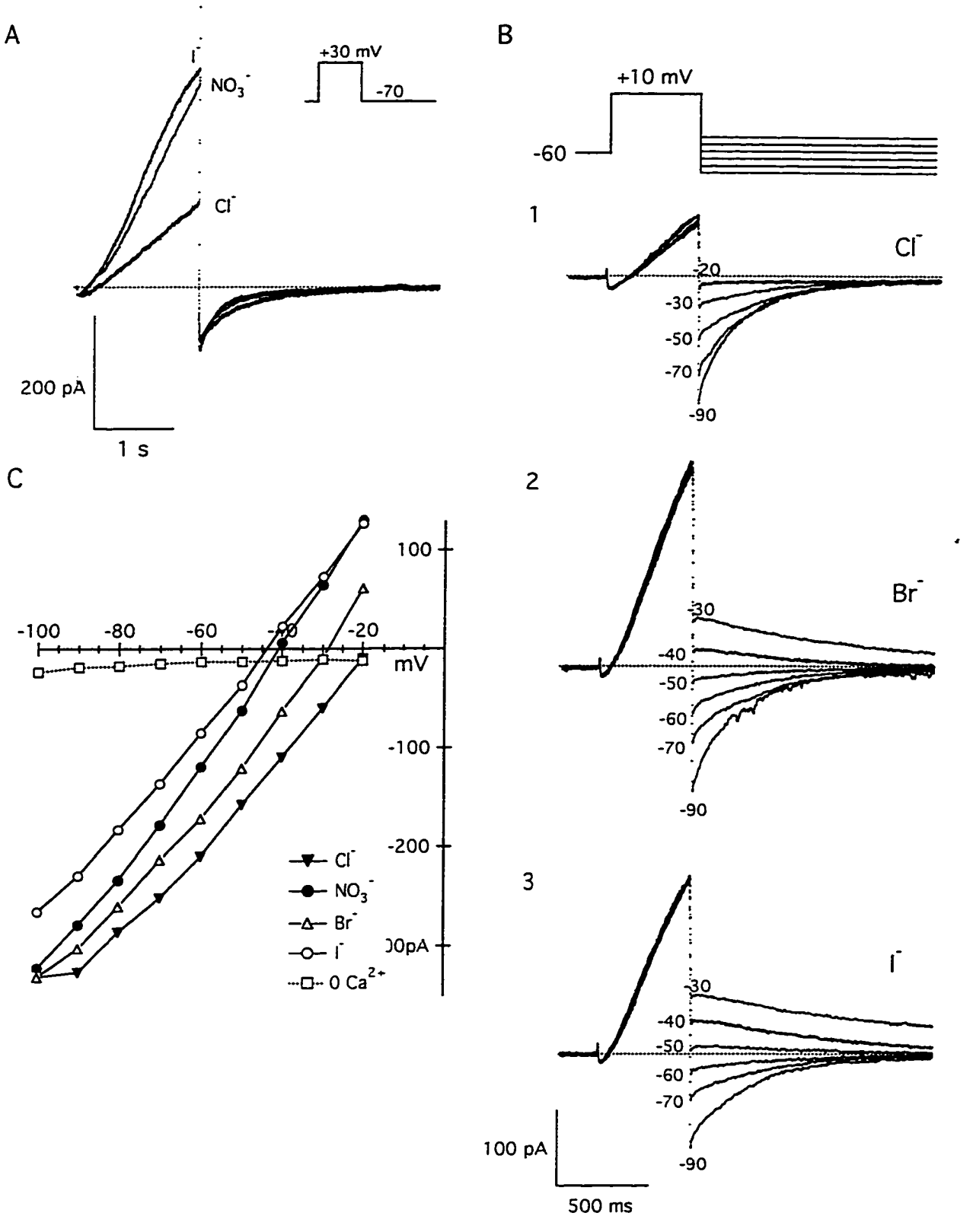


Fig. 13

anion was determined by fits with the Goldman-Hodgkin-Katz equation. Fig. 13B shows tail currents and Fig. 13C their corresponding I-V curves for the same cell in different anions. This gave the permeabilities I^-(n=3)>NO_3^- (n=8)>Br^-(n=5)>Cl^->acetate$^-$ (n=3). The permeability ratios calculated were 3.22>2.78>1.59.>1>0.67. This sequence corresponds to the Eisenman sequence I and is common to other chloride currents (Franciolini & Petris 1990). The same permeability sequence was obtained when 154 mM Cl^- (150 mM NaCl + 2 mM $CaCl_2$) in the external solution was replaced with the anion to make the conditions closer to biionic. When fluoride or isethionate were used to substitute 150 mM NaCl in the bath solution, the recording became unstable, therefore the permeabilities of these anions were not measured.

In addition to the above mentioned anions, the $I_{Cl(Ca)}$ was also permeable to SCN^- ion and impermeable to gluconate.

Muscarinic activation of the $I_{Cl(Ca)}$

So far we have described the properties of the $I_{Cl(Ca)}$ current that can be activated by Ca^{2+} influx from the exernal environment through the L-type Ca^{2+} channels. This same current can be activated when the source of Ca^{2+} is not external but the release from the intracellular stores. Muscarinic agonists have been demosntrated to cause an elevation of cytosolic Ca^{2+} in pancreatic β-cells and βTC-3 cells (Yada, Hamakawa & Yaekura 1995; Gromada & Dissing

1996). We tested whether our $I_{Cl(Ca)}$ could also be activated by muscarinic stimulation, via Ca^{2+} release. This was indeed the case. Application of 100 μM carbachol or muscarine reproducibly (n=15) activate a current which reverses at around -20 mV, close to the Cl^- equilibrium potential. Carbachol can activate this current both in the presence and absence of external Ca^{2+}. Atropine (10 μM abolishes the carbachol effect (n=2). This observation is consistent with carbachol acting to release Ca^{2+} from internal stores rather than modulating Ca^{2+} influx through the Ca^{2+} channels (Yada et al. 1995). Fig. 14 shows a $I_{Cl(Ca)}$ elicited by a ramp protocol in the presence of external Ca^{2+}. Upon carbachol application a large current is activated transiently reversing at -19 mV. The response desensitized as can be seen from the ramp-evoked current in the continued presence of carbachol (numbers 1-5). The carbachol response could be repeated after washout of the agonist for several minutes. In some cases the carbachol effect increasing the $I_{Cl(Ca)}$ after desensitization was followed by a reduction of the current at depolarized voltages (n=6). This effect may be due to depletion of the internal Ca^{2+} stores (see Discussion). To investigate the time course of the muscarinic activation of the $I_{Cl(Ca)}$ current the cell was clamped at -60 mV (Fig. 15). As shown in the inset, the cell had a very pronounced inwardly rectifying K^+ current. We took advantage of this to determine the time of arrival of carbachol (in different concentrations of K^+ solutions) and therefore the latency of the response. The bathing solution was B-30K^+ and therefore inward K^+ current can be seen. Carbachol was dissolved

Fig. 14. Carbachol potentiates the Ca^{2+}-dependent Cl^- current. Voltage ramps were generated every 10 s to obtain the I-V relation of the cell before and after 200 (M carbachol was applied. The numbers of the ramps denote their temporal sequence in the presence of carbachol. Desensitization is complete, trace 5 is almost identical with the control trace. Internal solution: B-I-K-sulphate, external solution: B-5K, 10 mM TEA, (2 mM Ca^{2+}).

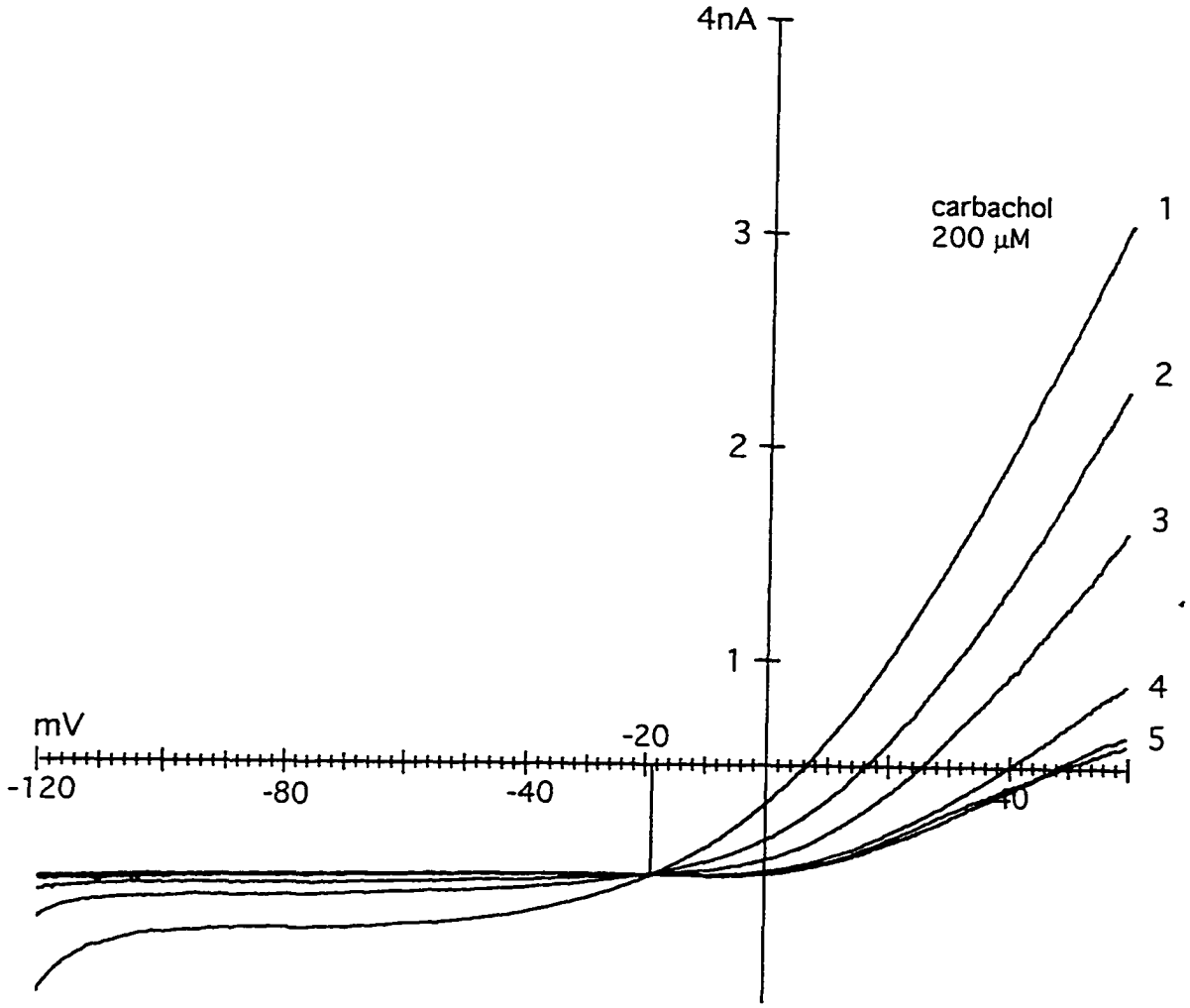


Fig.14

Fig. 15. The time course of carbachol response. The membrane was held at -60 mV to increase the driving force for Cl^- while keeping it low for K^+. Since external solutions (B-30K, 10 TEA and B-5K) contain 159 mM Cl^- , the calculated E_{Cl}=-23.7 mV. Also at this potential there is no influx of Ca^{2+} through the L-type Ca^{2+} channels. The inset shows an I-V for the same cell generated from a voltage ramp in B-5K, 10 TEA solution. The cell was bathed in B-30K solution and carbachol (50 μM) applied dissolved in B-5K. Note the change in the holding current when B-5K is introduced. Internal solution: B-I-K-sulphate.

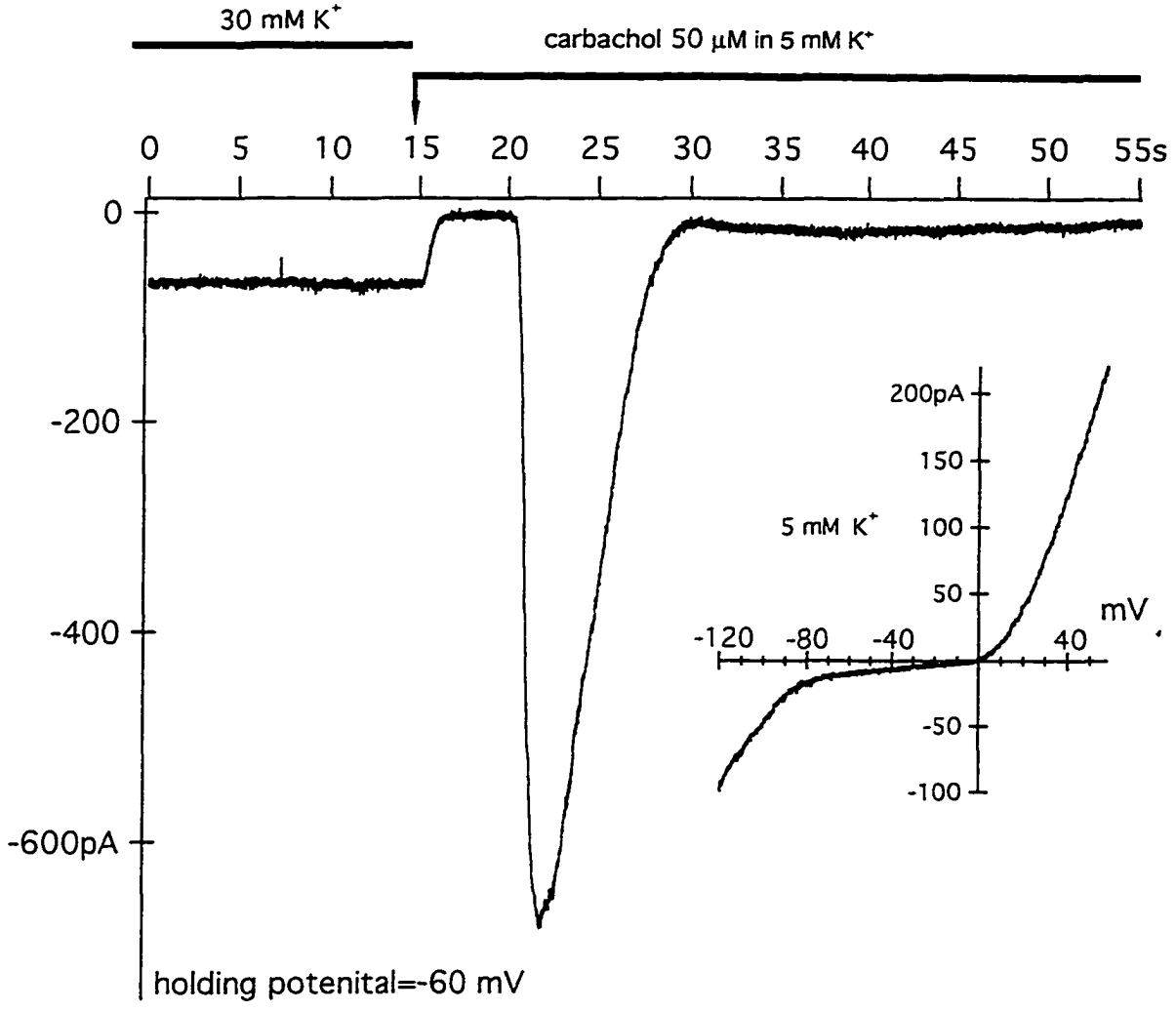


Fig.15

in B-5K$^+$ solution. Thus, upon arrival of the carbachol-containing solution the inward rectifier would conduct less current in the inward direction (E_K=-89 mV) and would provide the exact time of arrival of carbachol. After a delay of several seconds carbachol elicited an inward current. The response desensized in 8-9 seconds.

In another cell (Fig. 16) we could elicit the carbachol response over and over again after washout. In this case carbachol activated a large peak inward current followed by smaller amplitude oscillations. After removal of external Ca^{2+} carbachol could still activate the oscillating inward chloride current (panels 2,3,5). The irregularity of the oscillations in the presence of external Ca^{2+} was not consistent as very regular oscillations could be observed in normal external Ca^{2+} in other cells (n=5). Not all tested cells exhibited these oscillatory currents. In some of them (n=3) carbachol only activated a transient peak of $I_{Cl(Ca)}$ once. It is likely that the oscillations of the $I_{Cl(Ca)}$ are secondary to the intracellular Ca^{2+} oscillations rather than a property of the channel itself, since we did not observe this behavior during its activation by depolarizing voltage steps. Lack of effect of Na^+ on the $I_{Cl(Ca)}$ current.

Lack of effect of Na^+ on the $I_{Cl(Ca)}$ current.

It has been suggested that the depolarizing muscarinic response in pancreatic β-cells is dependent on extracellular Na^+ presence (Henquin,

Fig. 16. $I_{Cl(Ca)}$ oscillations elicited by carbachol in the presence and absence of external Ca^{2+}. The cell was held at -60 mV. Carbachol (100 μM in all cases) was applied when the extracellular solution contained 2 mM Ca^{2+} (1, 4) or contained 0 Ca^{2+} and 1 mM EGTA. The numebrs of panels reflect the sequence in which the applications of carbachol were done. Internal solution: B-I-K-sulphate, external solution: B-5K, 10 mM TEA.

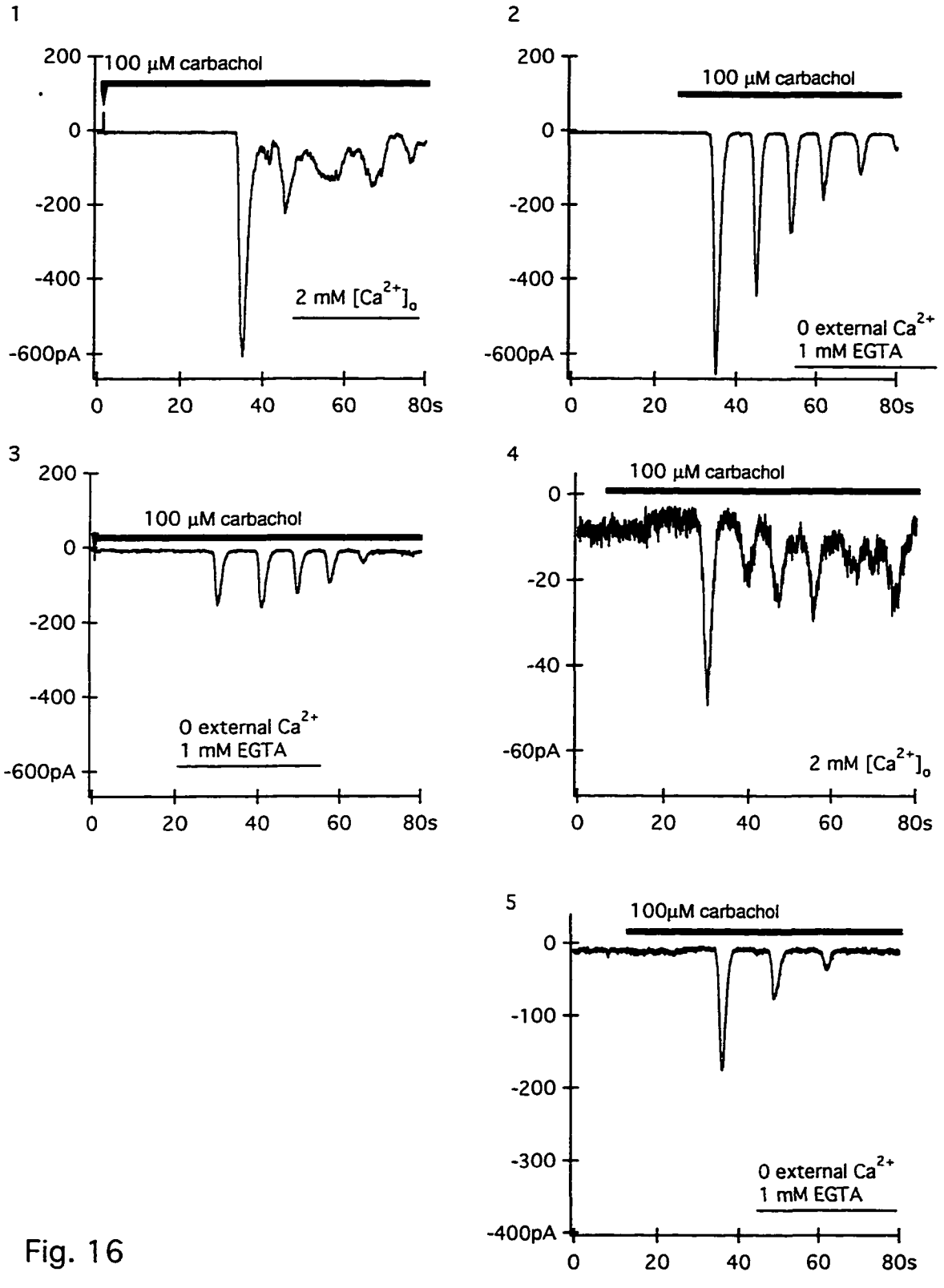


Fig. 16

Garcia, Bozem, Hermans & Nenquin 1988). Removal of external Na^+ and its replacement with N-methyl D-glucamine was without effect on the $I_{Cl(Ca)}$ amplitude and its reversal potential (n=4). Neither could the $I_{Cl(Ca)}$ be activated when the external divalents were replaced with Na^+ in the presence of Ca^{2+} chelator EGTA (Fig. 17A). The outward Cl^- current and the inward tail were both abolished and the inward current through Ca^{2+} channels carried by Na^+ was revealed. Removal of external divalents also caused an increase of leak as seen in panel B (cell membrane potential=-50 mV). Thus, neither external nor internal Na^+ could be linked to modulation/activation of the depolarizing $I_{Cl(Ca)}$ current in our cells. Whether the pipette contained Cs^+ or K^+ as the main cation was without effect on the reversal potential of the $I_{Cl(Ca)}$ as well.

Fig. 17. $I_{Cl(Ca)}$ cannot be activated by influx of Na^+ in the absence of Ca^{2+}. $I_{Cl(Ca)}$ was evoked by a long depolarizing step to 0 mV (A). Removal of external Ca^{2+} and addition of EGTA 1 mM prevented the current activation, although Na^+ ions could flow in through the L-type Ca^{2+} channels under these conditions. Internal solution: B-I-K-sulphate, external: 5 mM K^+.

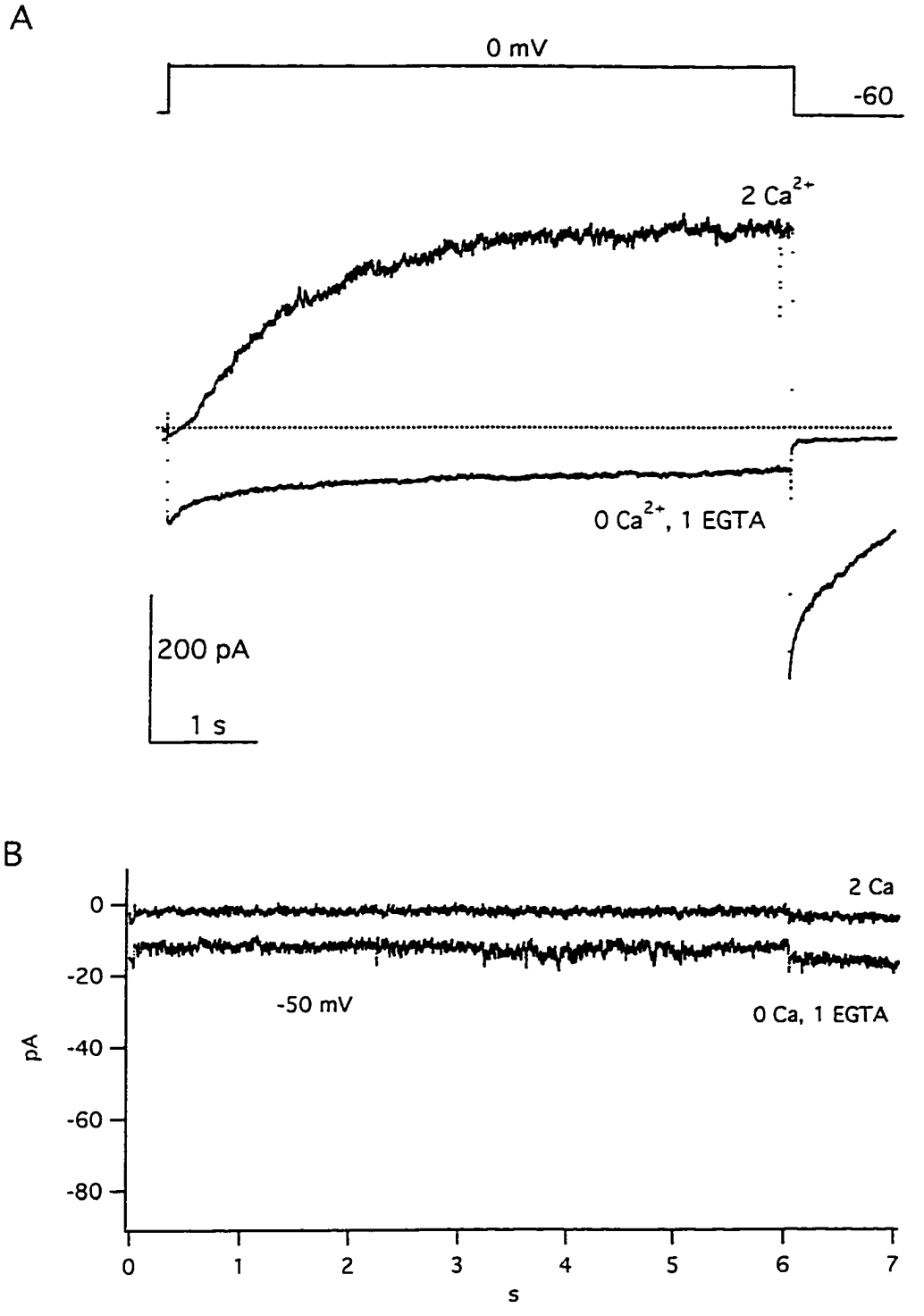


Fig. 17

Chapter 5

The Ca^{2+} -activated K^+ current in βTC-3 and pig islet cells

Depolarizing voltage steps activate an outward current dependent on extracellular Ca^{2+}

In the presence of 10 mM external TEA depolarizing steps from a holding -60 mV potential elicited slow outward currents (Fig.18A). Upon repolarization to -60 mV slow outward tail currents were observed. The outward tail currents were recorded at -60 mV to distinguish the tails from those of the $I_{Cl(Ca)}$ which can also be found in βTC-3 cells and would be inward (see Chapter 4, Fig. 6) and at this potential would show an inward direction. Addition of 100 μM Cd^{2+} to the bath abolished reversibly both the "onset" and the tail currents (Fig. 18 B,C). Removal of external calcium or its replacement with Mg^{2+} had the same effect. A current-voltage relationship of the peak "onset" (Fig. 18B) and tail current (Fig. 18C) is shown. The I-Vs are bell shaped and reminiscent of the calcium current I-V found in these cells (Chapter 3, Fig. 2). The correlation of the I-V shape of this current with I_{Ca} and the dependence on Ca^{2+} influx indicate that this outward current is calcium-activated.

The Ca^{2+}-activated outward current increases with longer depolarizing steps

Fig. 18. Depolarizing voltage steps activate an outward current which requires influx of external Ca^{2+}. **A.** Left panel: 800 ms long depolarizing voltage steps were applied from a holding potential of -60 mV and the evoked outward currents are shown in normal external solution (2 mM Ca^{2+}). Middle panel: 100 μM Cd^{2+} application in the presence of 2 mM Ca^{2+} abolished the slowly activating outward currents as well as the slow tail currents. The panel on the right shows the reversal of Cd^{2+} block after washout. The current voltage plot of "onset" (**B**) and tail (**C**) current generated from the traces shown in A. Both "onset" and tail current amplitudes are plotted against the test potential.

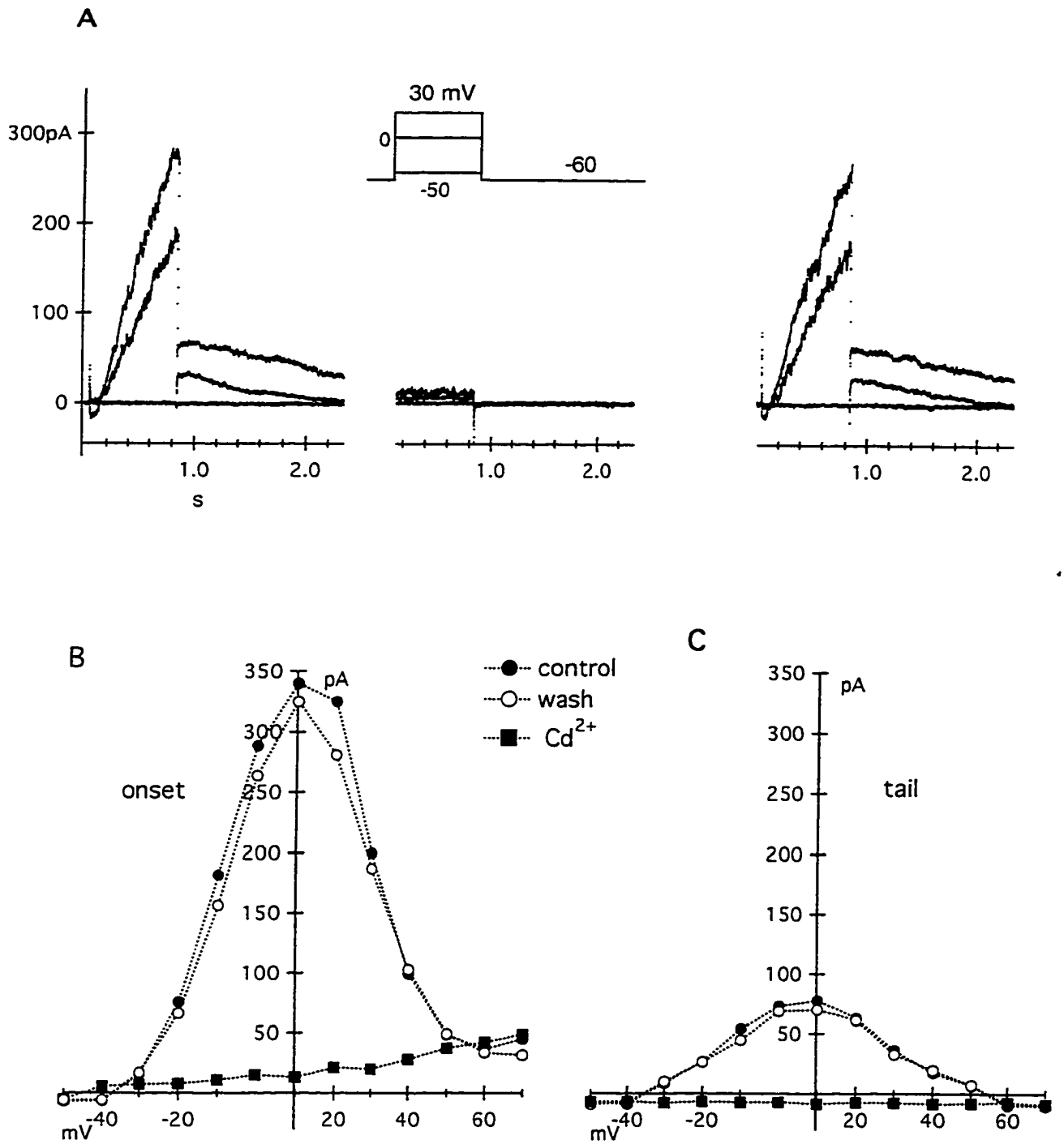


Fig. 18

If the depolarization activated "onset" current and the tail current are activated by calcium elevation one would expect to see larger outward currents when increasingly longer depolarizing steps are applied by providing more Ca^{2+} influx, thereby activating more channels. To test this the "envelope" protocol shown in Fig. 19A was used. Progressively longer steps to +10 mV were applied and the voltage was returned to -50 mV after each step to record a tail current. The +10 mV step value was chosen to maximize I_{Ca} while the -50 mV repolarization value was chosen to increase the outward tail current size. As shown previously (see Chapter 3) no significant inactivation of I_{Ca} occurs during voltage steps up to 2 seconds long, therefore in such an "envelope" experiment Ca^{2+} influx continues during all steps. Longer voltage steps elicit progressively longer outward onset and tail currents which are correlated linearly (not shown). Such linear correlation strongly suggests that both "onset" and tail currents are carried by the same ion channel species. Fig. 19 inset shows that in the absence of external Ca^{2+}, the Ca^{2+}-activated current is abolished.

Strontium but not barium substitute for calcium in activating the Ca^{2+}-dependent outward current

As was demonstrated earlier (Chapter 3) and in agreement with other studies, the L-like calcium channels in βTC-3 cells are permeable to both Ba^{2+} and to Sr^{2+}. We tested whether any of these permeant divalent ions would

Fig. 19. Progressively longer depolarizing voltage steps activate larger Ca^{2+}-activated outward currents. Voltage steps of increasing durations to +10 mV ("envelope protocol") evoke correspondingly larger outward currents followed by larger and slower tail currents. Inset: the same votage protocol applied in the absence of external Ca^{2+}. The outward currents are completely abolished.

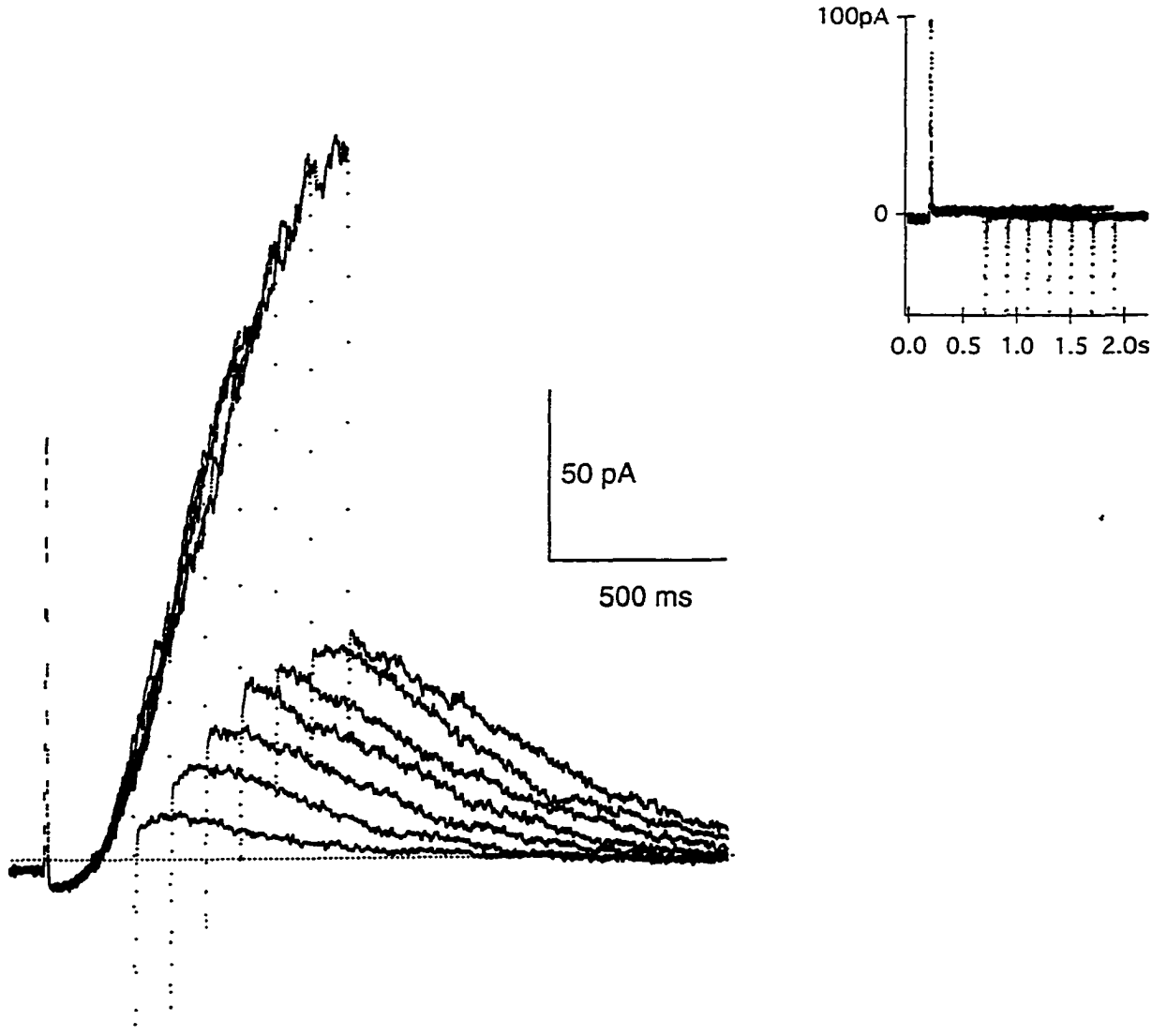


Fig. 19

mimic Ca^{2+} in activating the outward current. Replacing the external 2 mM Ca^{2+} with equimolar Sr^{2+} and Ba^{2+}. As seen in Fig. 20A a depolarizing step in external Sr^{2+} activated an outward current of similar size to the control but in external Ba^{2+} it failed to do so. Increasing external Ba^{2+} to 20 mM was ineffective. As with the $I_{Cl(Ca)}$ (Kozak & Logothetis 1997) Sr^{2+} but not Ba^{2+} could substitute for Ca2+ in activating the outward current (Fig. 20B). Sr^{2+} seems to be a more potent activator for this K(Ca) current compared with the Ca^{2+}-dependent Cl^- current, which under similar conditions showed relatively reduced amplitude (Kozak & Logothetis 1997). The slow Ca^{2+}-activated current is K^+-selective. In order to determine the nature of the outward Ca^{2+}-dependent current its reversal potential was measured as a function of the external K^+ concentration. As shown in Fig. 21A, the membrane potential was stepped to a depolarizing voltage (+5 mV) followed by a series of steps to various hyperpolarized potentials (-110 to -40 mV). The same paradigm was repeated in the presence of 100 μM Cd^{2+} and subtracted from the control traces. The peak of the tail current was measured 15 ms following the repolarization step. Fig. 21B shows the tail I-V plot generated in this fashion. The reversal potential of the current determined from the I-V plot was -83 mV (n=5). This value is close to the calculated Nernst potential for K^+, -89 mV. At an external potassium concentration of 30 mM (Fig. 21A, right) as compared to 5 mM there was a positive shift of the reversal potential of the Ca^{2+}-dependent current (+45.15 mV), as predicted by the Nernst equation for a K^+-selective

Fig. 20. The effects of substituting the external Ca^{2+} with Sr^{2+} or Ba^{2+} on the Ca^{2+}-activated current. **A.** Outward currents were evoked by steps to +10 mv in the presence of 2 mM external Ca^{2+} (trace 1), Sr^{2+} (trace 2). **B.** External Ca^{2+} is replaced with 20 mM Ba^{2+}. Ba^{2+} was ineffective in activating the outward current. The inward Ca^{2}+ current (carried by Ba^{2+}) can be seen. Thus, Sr^{2+} but not Ba^{2+} can substitute for Ca^{2+} in activating the outward current.

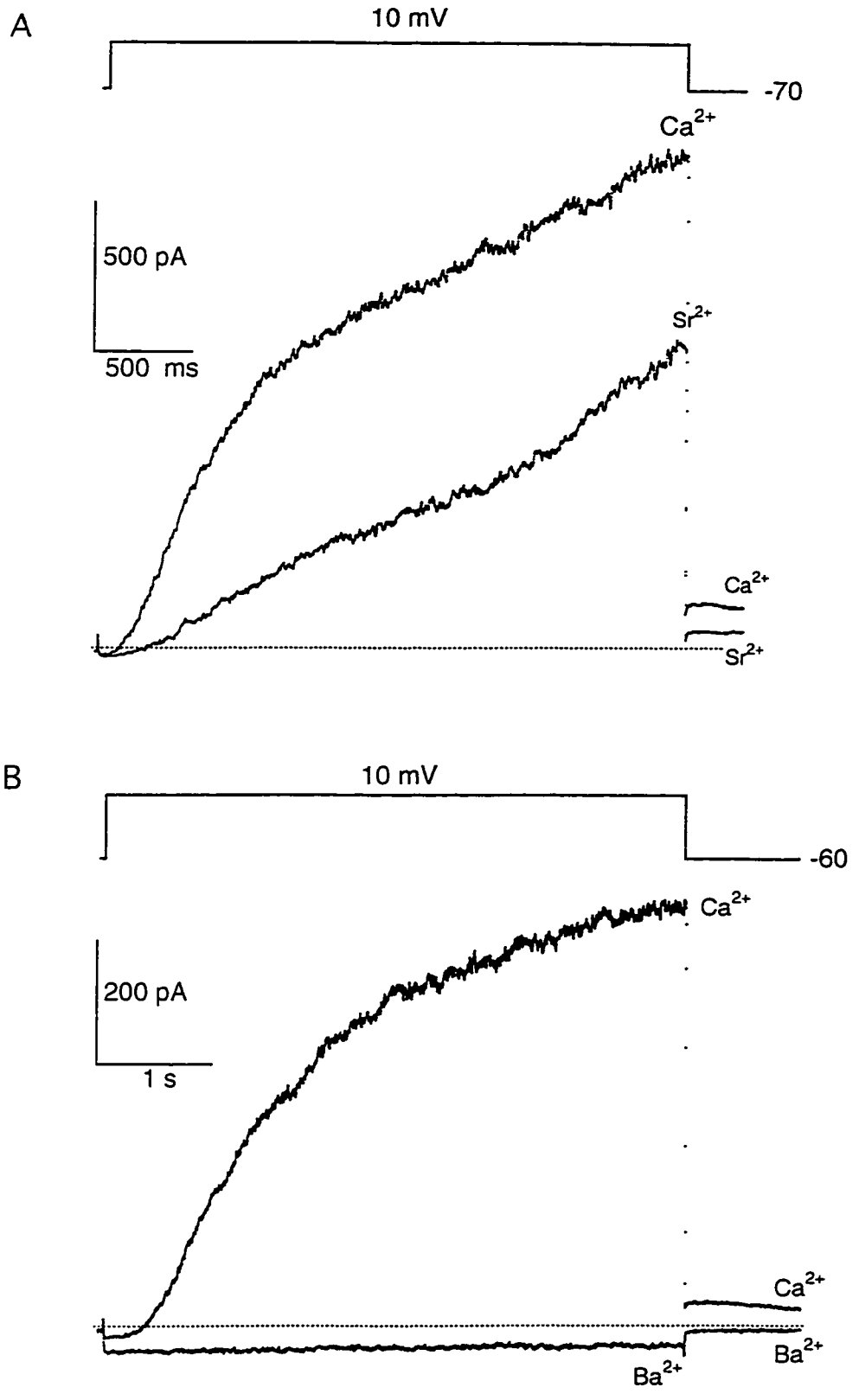


Fig. 20

channel. In the experiments with external K^+ increase either the Na^+ concentration was reduced or a high K^+ solution was used which contained N-methyl D-glucamine instead of Na^+. No difference in the reversal potential values were found when Na+ concentrations were altered (n=3). The reversal potential did not vary when the internal solution contained gluconate instead of sulphate as the anion (see *Methods*). Based on these results we concluded that this current constitutes a Ca^{2+}-dependent K^+ current ($I_{K(Ca)}$). The selectivity sequence of permeant ions through $I_{K(Ca)}$. In order to determine the ionic selectivity sequence of this current the external 30 mM K^+ was replaced with equimolar salt of another cation. The reversal potential was then determined in various external solutions and compared to that in 30 mM K^+ solution. As seen in Fig. 21C, K^+, Rb^+ and Tl^+ were permeant through the channel, whereas Cs^+ was not. The selectivity sequence was determined using a modified Nernst equation (Hille 1992) for biionic conditions, which gave the following permeability ratios: $Tl^+ > K^+ > Rb^+ > NH_4^+$. Na^+ was not measurably permeant. The reversal potential values for various permeant ions were not affected by the presence of external Na^+ compared to N-methyl D-glucamine.

The blockers of the $I_{K(Ca)}$ current

Mouse pancreatic β-cells have been shown previously to contain high conductance Ca^{2+}-dependent K^+ channels (Maxi K^+), sensitive to block by external TEA (Tabcharani & Misler 1989). We also observed TEA block of the

Fig. 21. The Ca^{2+}-activated current reversal potential value is sensitive to changes in external K^+ concentration. The ionic selectivity sequence. **A.** Tail currents were evoked at different membrane potentials following a depolarizing step to step +5 mV. The external potassium was 5 mM (left) and 30 mM (right). **B.** The peak tail current amplitude is plotted against the tail voltage value to generate a tail current-voltage relation, from which the reversal potential values can be obtained in the three K^+ external concentrations (5, 30, 100 mM). **C.** The ionic selectivity sequence of the slow K(Ca) current derived from the reversal potential measurements. The reversal potential of the Cd^{2+}-sensitive current was measured in an external solution containing 30 mM K^+ and compared to that in solutions containing 30 mM of the monovalent cation in question (Tl^+, Rb^+). The individual points are fitted with a 3rd degree polynomial.

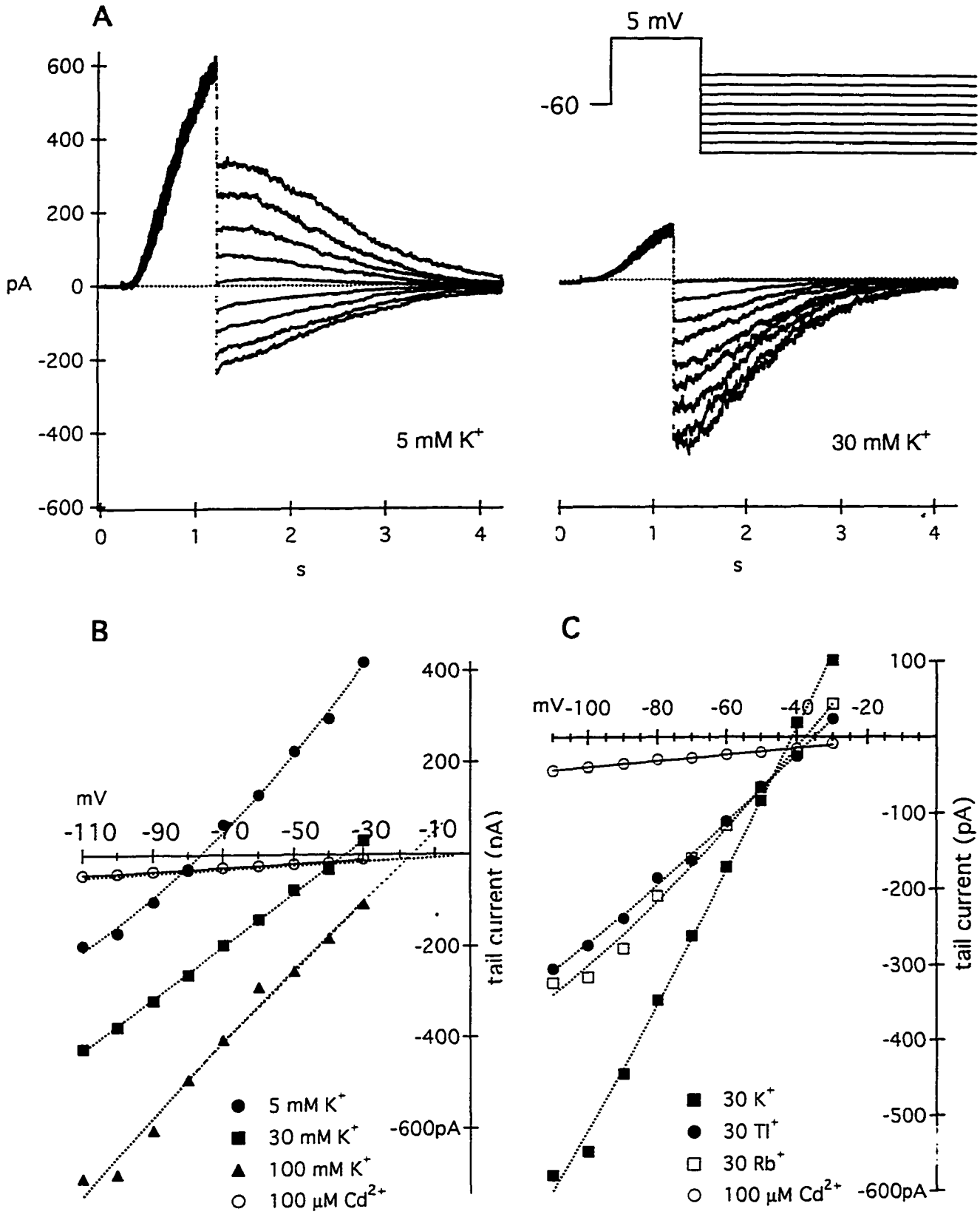


Fig. 21

Maxi K^+ channels in βTC-3 cells (not shown) and have used 10 mM TEA in our external solutions in order to block these channels. The slow time course of activation and deactivation of βTC-3 $I_{K(Ca)}$ suggested an SK-like current described extensively in other secretory cells (Park 1994). This current has been shown to be sensitive to block by bee venom toxin apamin and tubocurarine. 200 nM apamin and 2 μM D-tubocurarine had no effect on βTC-3 $I_{K(Ca)}$ current (n=4) (data not shown).

The insensitivity of $I_{K(Ca)}$ to these drugs is consistent with previous studies in islets and single β-cells (Lebrun, Atwater, Claret, Malaisse & Herschuelz 1983; Henquin, Garrino, Nenquin, Paolisso & Hermans 1985; Ämmälä et al. 1993), which point out the lack of apamin-sensitive potassium conductance in these cells.

There are reports of charybdotoxin blocking the small conductance Ca^{2+}-activated K^+ current in various tissues (reviewed in Latorre, Oberhauser, Labarca & Alvarez 1989). As shown in Fig. 22A increasing concentrations of charybdotoxin blocked the $I_{K(Ca)}$. 100 nM (the highest concentration tested) blocked on average 80% of the current. Fig. 22B shows the charybdotoxin effect on the $I_{K(Ca)}$ I-V. The block was reversible and voltage-independent.

Scyllatoxin (leiurotoxin I) (Castle & Strong 1986; Castle, Haylett & Jenkinson 1989), which is known to block the apamin-sensitive channel in other tissues, was also ineffective in blocking this current (up to 200 nM). Fig.

Fig. 22. The slow, Ca^{2+}-activated K^+ current in βTC-3 cells is sensitive to block by charybdotoxin **A.** The TEA-insensitive Ca^{2+}-activated K^+ current was blocked by increasing concentrations of charybdotoxin (10 nM and 100 nM). **B.** shown are the effects of 100 nM ChTX on the peak outward current I-V. The inset shows a current trace evoked by a step to 0 mV (1) and block by 100 nM ChTX (2).

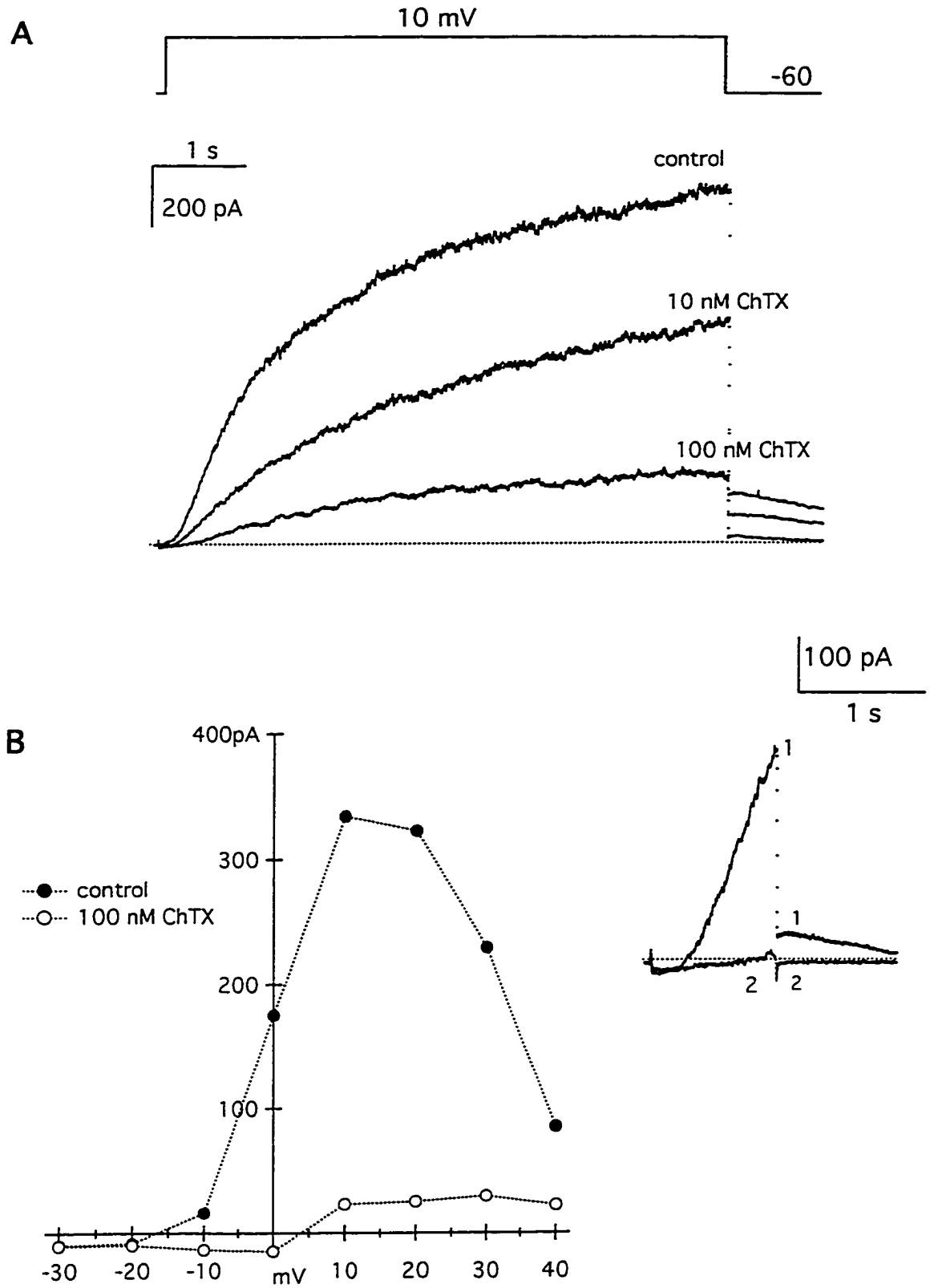


Fig. 22

23 shows that 40 nM scyllatoxin failed to block the $I_{K(Ca)}$ (panel A), whereas 10 and 100 nM ChTX caused a marked block (44.3% and 84.6%) of the Cd^{2+}-sensitive current (panel B).

Iberiotoxin (IbTX), another scorpion toxin, is closely related to charybdotoxin and selectively blocks the Maxi K^+ channel. 100 nM iberiotoxin did not affect the $I_{K(Ca)}$ current (n=3). IbTX did however block the Maxi K^+ channels as seen (not shown). In a perforated-patch whole cell mode and in the absence of TEA from the external solutions, some cells showed lack of any other channels except Maxi K^+. These cells were advantageous for testing the efficiency of the toxins. In this case, a perforated whole-cell could be treated like an outside-out patch recording, where the extracellular side of the channel is facing the bath. Therefore toxins could be applied in the bath.

100 nM Kaliotoxin, which is also known to block the Maxi K^+ channel did not affect the TEA-insensitive βTC-3 cell $I_{K(Ca)}$ current (n=4).

It has been shown previously that quinine blocks a Ca^{2+}-dependent K^+ conductance in β-cells (Atwater, Dawson, Ribalet & Rojas 1979; Ashcrof & Rorsman 1991). In this study 100 μM quinine abolished the $I_{K(Ca)}$ current completely (n=4). Quinine however, is known to inhibit other potassium conductances in β-cells such as the inward rectifier and the delayed rectifier (Findlay, Dunne, Ullrich, Wollheim & Petersen 1985; Bokvist et al. 1990; Bokvist, Rorsman & Smith 1990), which was also the case in βTC-3 cells (J. A. K. unpublished observations). Cs^+ ions, which are known to block various

Fig. 23. Lack of scyllatoxin (leiurotoxin) effect on the slow Ca^{2+} activated K^+ curent. **A.** $I_{K(Ca)}$ evoked by a 1.5 s long step to 10 mV from holding potential of -60 mV. 40 nM scyllatoxin did not affect the "onset" or the tail currents which could be blocked by 100 μM Cd^{2+}. **B.** In the same cell increasing concentrations of ChTX blocked both the "onset" and tail outward currents: 10 nM blocked 44.3% and 100 nM-84.6% of the total Cd^{2+}-sensitive current respectively. Internal solution: B-I-K-sulphate, external solution: B-5K, 10 mM TEA.

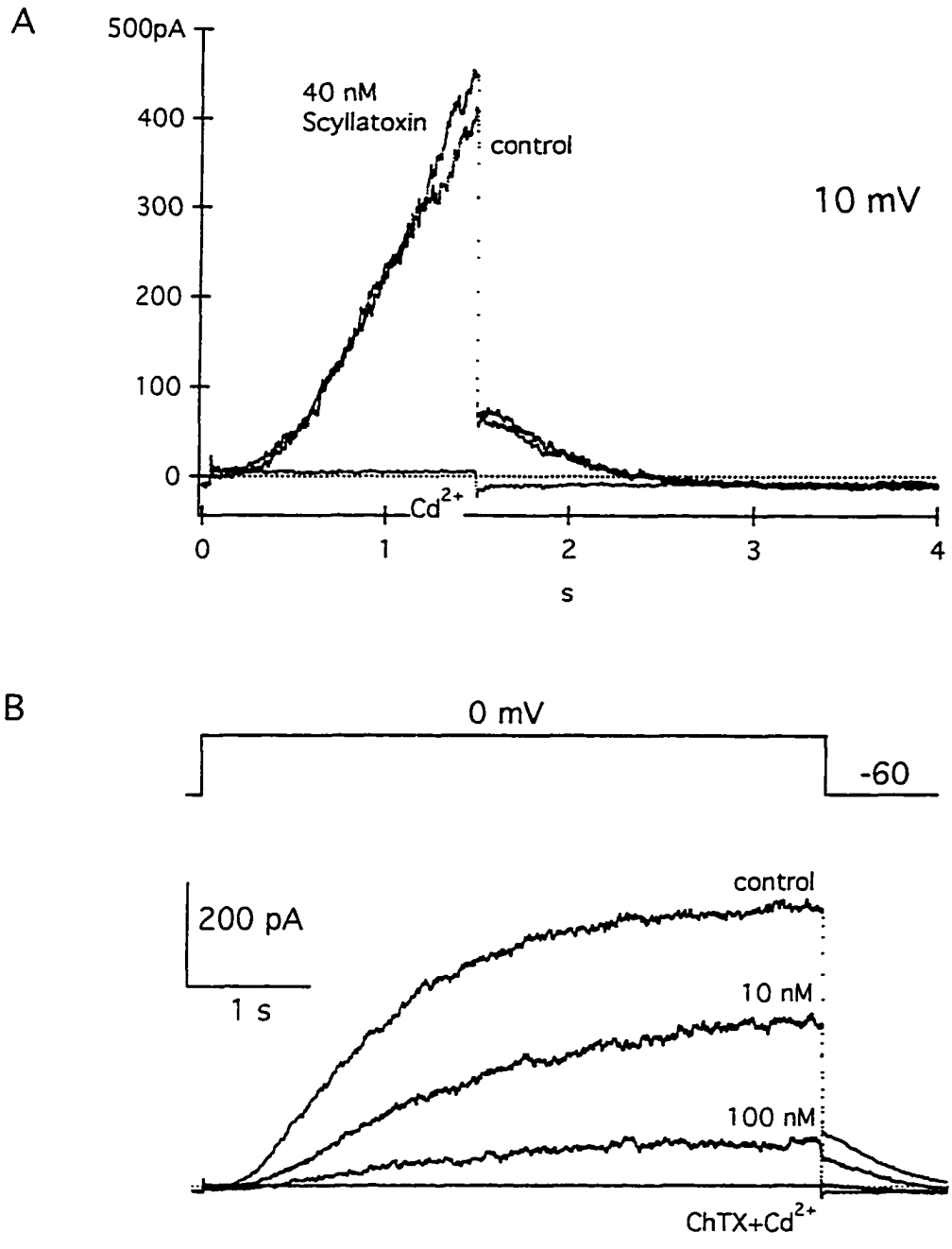


Fig. 23

potassium channels, such as intermediate-conductance Ca^{2+}-activated channels (Schwarz & Passow 1983) were ineffective in blocking the potassium current through $I_{K(Ca)}$ (1 -2 mM).

Clotrimazole, a cytochrome P-450 pathway inhibitor, was recently shown to be a blocker of erythrocyte K(Ca) (Brugnara, De Franceschi & Alper 1993; Brugnara, Armsby, De Franceschi, Crest, Martin-Eauclaire & Alper 1995) and Maxi K^+ channels in smooth muscle (Rittenhouse, Parker, Brugnara, Morgan & Alper 1997). In Fig. 24 shows a cell which was "mixed", expressing both $I_{K(Ca)}$ and $I_{Cl(Ca)}$. These two currents can be discerned from the two components in the tail current at -55 mV, the faster, inward one, which represents Cl^- current contribution and the slower, outward one, characteristic of the K^+ current (trace 1). At 1 μM, clotrimazole reduced the outward "onset" current (trace 2) and blocked the tail current's outward, slow component. In the presence of clotrimazole, 100 nM ChTX blocked an additional outward current and (trace 3) revealed mostly the inward tail current. Higher concentrations of clotrimazole were not tried to avoid compounded effects due to P-450 cascade activation. Clotrimazole block of βTC-3 cell $I_{K(Ca)}$ was estimated to be >60% (n=4).

Niflumic acid is a potent activator of the $I_{K(Ca)}$

Fig. 24. Clotrimazole, an imidazole, blocks the Ca^{2+}-activated K^+ current. $I_{K(Ca)}$ was evoked in the control solution (trace 1), in the presence of 1 μM clotimazole (trace 2) and in the presence of clotrimazole and 100 nM ChTX. Internal solution: B-I-K-sulphate, external solution: B-5K, 0 TEA.

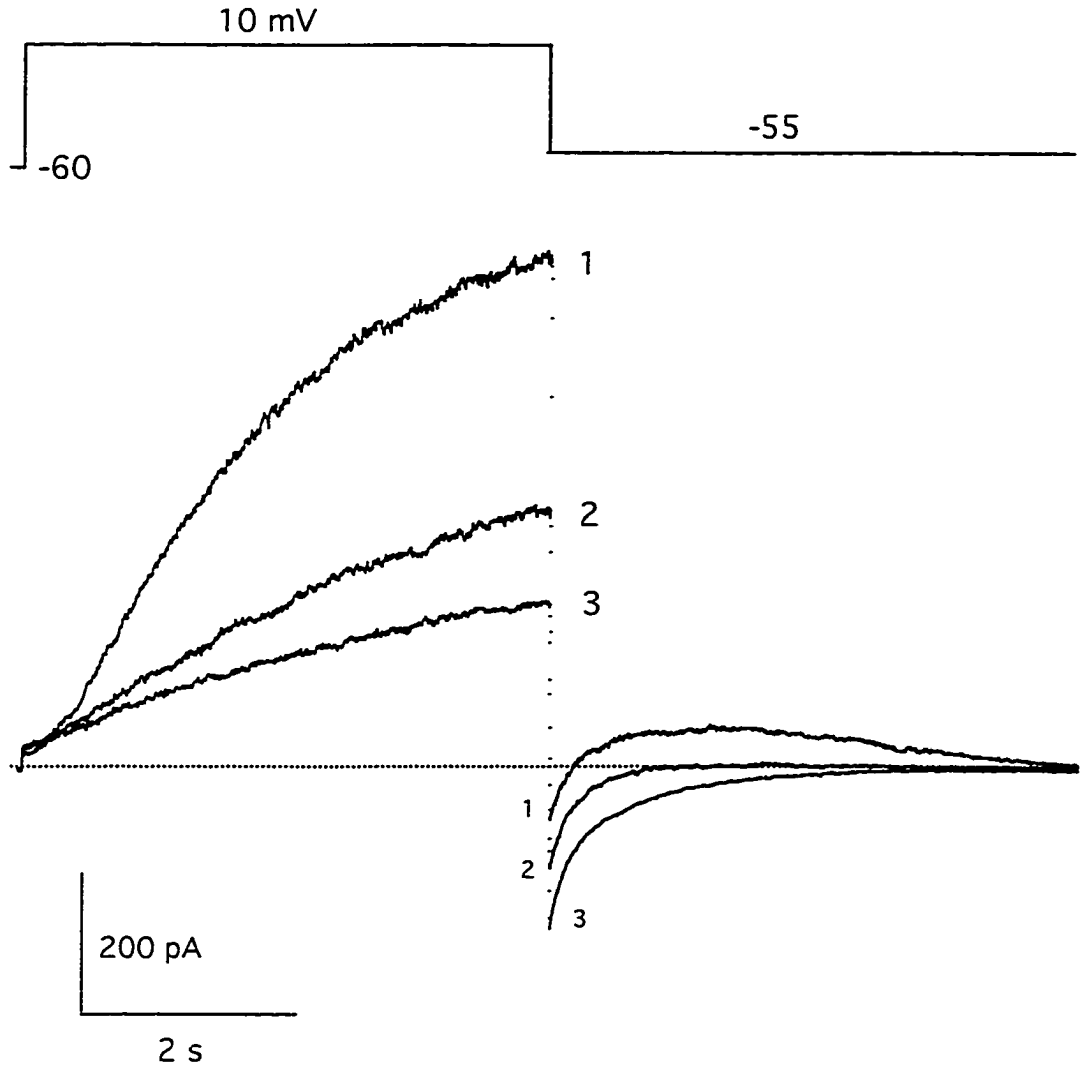


Fig. 24

In Chapter 4 it was shown that niflumic acid reversibly blocks the Ca^{2+}-dependent Cl^- current in βTC-3 cells at micromolar concentrations. Initially, we tried to separate the $I_{K(Ca)}$ from the $I_{Cl(Ca)}$ in cells containing both currents, by adding niflumic acid to the bath. Unexpectedly it was discovered that 10-20 μM niflumic acid could activate the $I_{K(Ca)}$ in addition to blocking the chloride current. Fig. 25A shows the effect of 10 μM niflumic acid on the $I_{K(Ca)}$ activated by a depolarizing step to +10 mV. Niflumic acid markedly potentiated both the "onset" and the tail currents, in a fully reversible manner (not shown) and its effect did not desensitize throughout its application. The niflumic acid effect on the I-V is shown in Fig. 25B. There was no apparent voltage-dependence of potentiation of the current at voltages where the current can be activated by calcium influx via the L-like channels. Niflumic acid failed to activate the $I_{K(Ca)}$ when the external solution contained 100 μM Cd^{2+} (data not shown).

Separation of K^+ and Cl^- components in a "mixed" cell

As mentioned above, many βTC-3 cells studied exhibited both Ca^{2+}-dependent conductances. This was apparent from the direction and the shape of the tail current as well as the reversal potential of the Ca^{2+}-sensitive current, which occured between the equilibrium potentials of K^+ and Cl^-. The pharmacological tools, the inhibitors and activator of the K(Ca) and Cl(Ca) currents can be applied to separate these two currents. In Fig. 26 a recording

Fig. 25. Micromolar concentrations of niflumic acid potentiate the slow Ca^{2+}-activated K^+ current. **A.** 10 μM niflumic acid increases the depolarization evoked "onset" and the repolarization-accompanied tail current. **B.** 10 and 20 μM niflumic acid applications progressively increase the $I_{K(Ca)}$ at various membrane potentials. The niflumic acid effect is not voltage dependent. The I-V curves were generated from the same cell as in A.

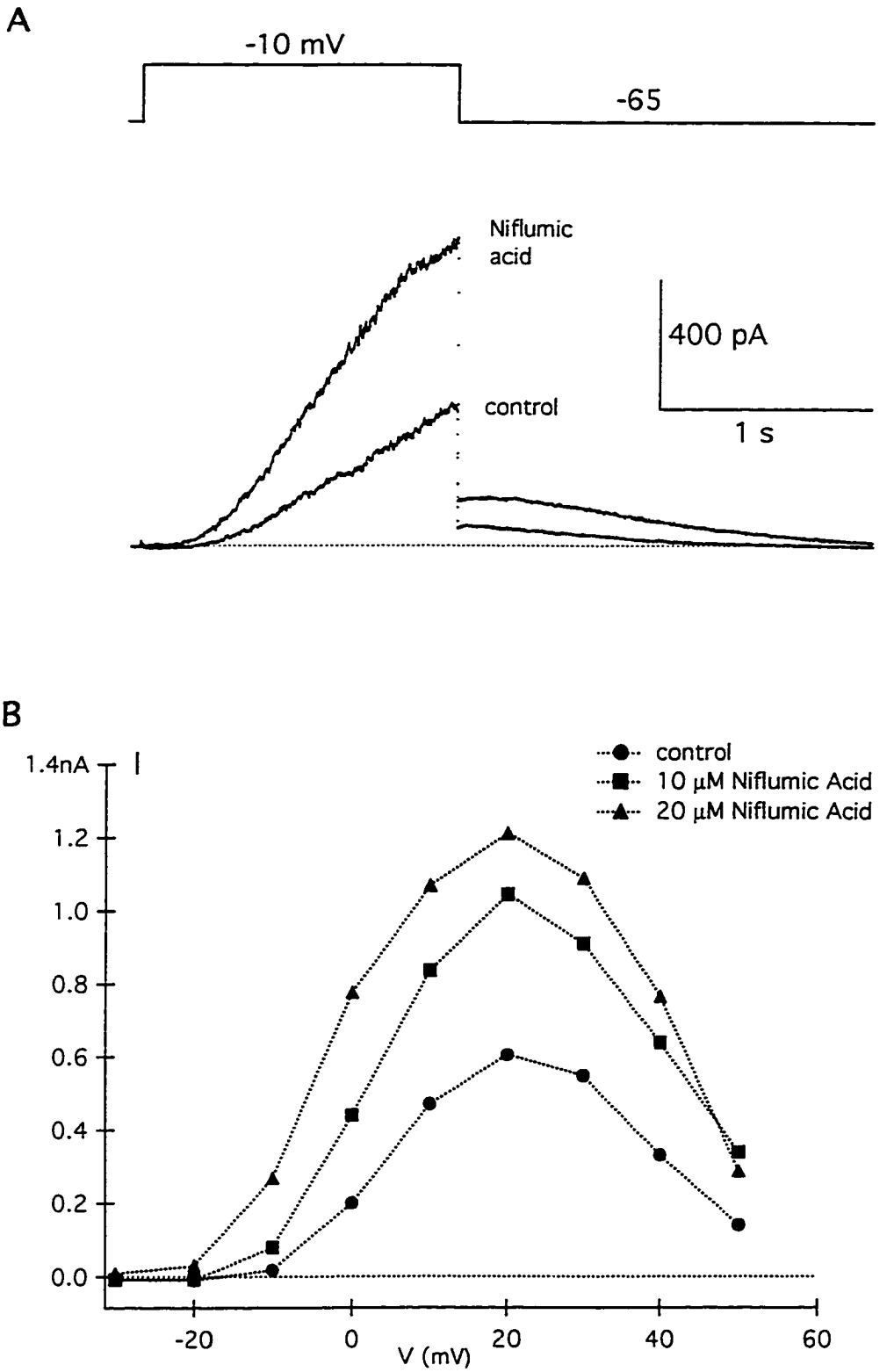


Fig. 25

from one such "mixed" cell is presented. The reversal potential of the Ca^{2+}-sensitive current is determined from the tail potentials (panel A). In panel B, the tail current-voltage relation is presented. The control reversal potential is approximately -42 mV. After applying 100 nM charybdotoxin (panel A, middle), the reversal potential shifts to a more positive value (B) as expected if most of the remaining current is carried by Cl^-. The charybdotoxin effect was fully reversed by wash. Subsequently niflumic acid (10 μM) was added (panel A, right) which produced a reversal potential shift towards more negative values (panel B). The current amplitude was decreased in the inward and increased in the outward direction. This reduction in the inward current is consistent with blockage of the Cl(Ca) current, which conducts primarily in the inward direction (due to large driving force). The increase in the outward current is consistent with an increase in the outward K(Ca) current by niflumic acid. The decay speed of the tail current is increased in ChTX (mostly Cl(Ca)) and slowed down in niflumic acid (mostly K(Ca)). Thus, ChTX and niflumic acid are useful for separation of the two Ca^{2+}-sensitive currents in βTC-3 cells.

The kinetics of activation and deactivation: the Bay K effect

As seen in Figs 18, 20, 22 the time course of activation and deactivation (the tail current) of the $I_{K(Ca)}$ is very slow (seconds). We hypothesized that both of these rates may reflect the speed of calcium accumulation caused by Ca^{2+}

Fig. 26. Separation of K^+ and Cl^- components of the Ca^{2+}-dependent current. The reversal potential of the whole Ca^{2+}-dependent current was determined from tail currents recorded using the standard protocol: from holding potential of -70 mV the cell was depolarized to 0 mV for 500 ms then stepped to various potentials to record the tail currents. **A.** the reversal potential of the Ca^{2+}-dependent currents in normal external solution (B-5K, 10 TEA), in the presence of 100 μM charybdotoxin and in the presence of 10 μM niflumic acid. Niflumic acid was added after the ChTX effect was reversed by washout. **B.** The tail current-voltage relation obtained from the recordings shown in A. The internal solution was B-I-K-sulphate.

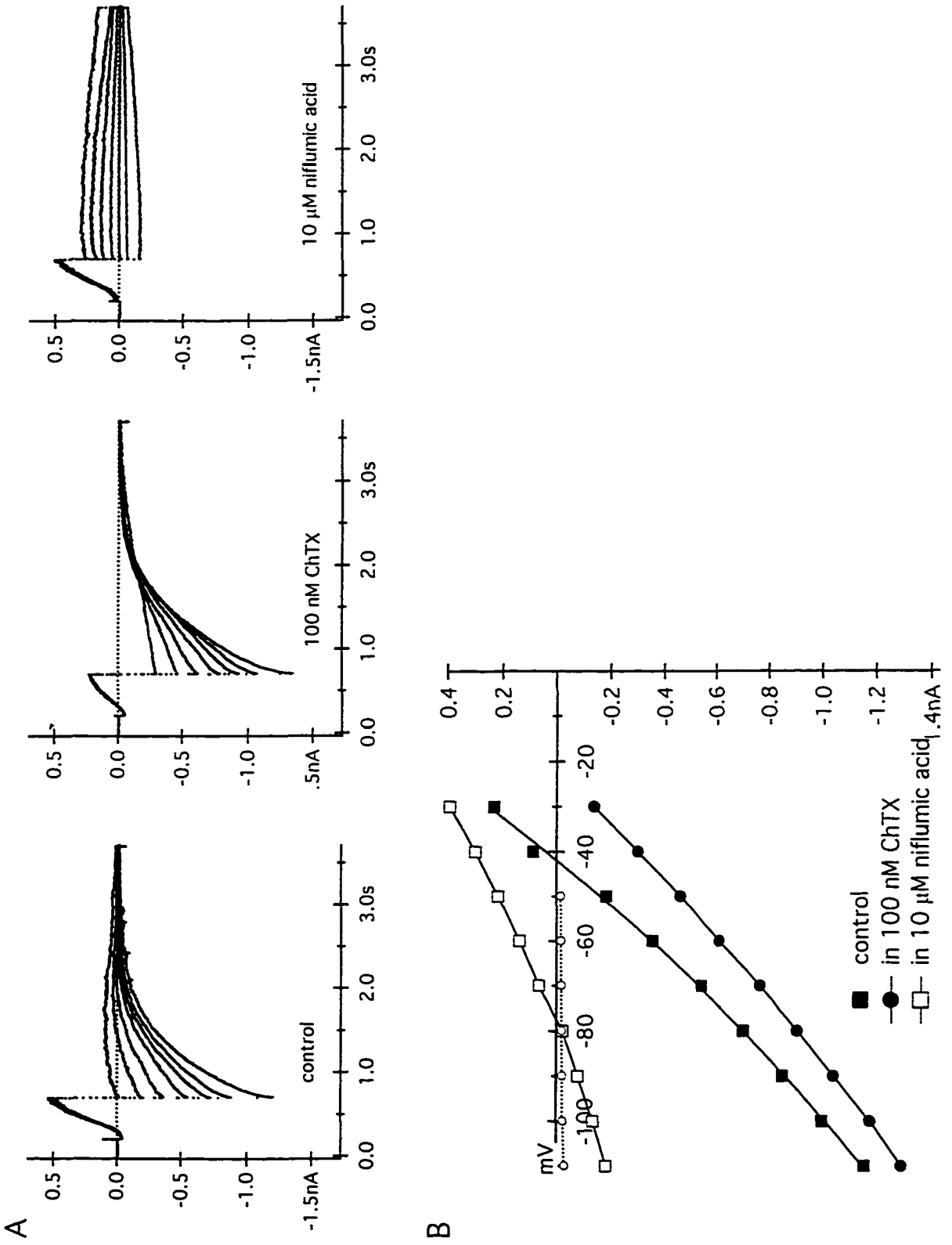


Fig. 26

influx through VDCCs (activation) and the speed of Ca^{2+} buffering (deactivation), rather than the behavior of the channel gate itself. Bay K 8644 was used, a dihydropyridine activator of L-type Ca^{2+} channels (Lebrun & Atwater 1985), to test this hypothesis. Bay K is expected to increase the rate of the channel activation, because it will increase the size of the inward Ca^{2+} current and therefore the amount of Ca^{2+} influx. It is also expected to slow down the tail current (the deactivation), as more time will be required to buffer and pump out increased amounts of Ca^{2+}. As shown in Fig. 27A, Bay K (1 μM) increased the amplitude of the $I_{K(Ca)}$ and the tail current. When the control peak outward currents are scaled appropriately to match those in the presence of Bay K (Fig. 27B), it can be seen that Bay K increases the activation rate and slows the tail current (deactivation). These results suggest, therefore, that the speed of activation and deactivation are dependent on intracellular Ca^{2+}. The dihydropyridine Ca^{2+} channel blocker nifedipine (100 nM) as well as the phenylalkylamine verapamil (200 μM) reversibly blocked the activation of the $I_{K(Ca)}$ by depolarizing voltage steps (n=4, data not shown).

Muscarinic activation of the $I_{K(Ca)}$ in βTC-3 cells

Acetylcholine (acting through muscarinic receptors) has been shown to activate Ca^{2+}-activated K^+ channels in lacrimal cells (Marty 1989) by releasing Ca^{2+} from intracellular stores. We tested whether carbachol and muscarine

Fig. 27. The speed of onset and deactivation of the current are affected by the L-type Ca^{2+}-channel agonist Bay K. **A.** Ca^{2+}-activated K^+ current was evoked by a long depolarizing pulse in the presence and absence of Bay K (1 μM). The amplitude of the "onset" and the tail current is increased. **B.** The same recording as in A with the control trace scaled to match the maximum amplitude of that in the presence of Bay K. The faster time course of the activation (onset) and slower time course of deactivation (tail) in Bay K are observed.

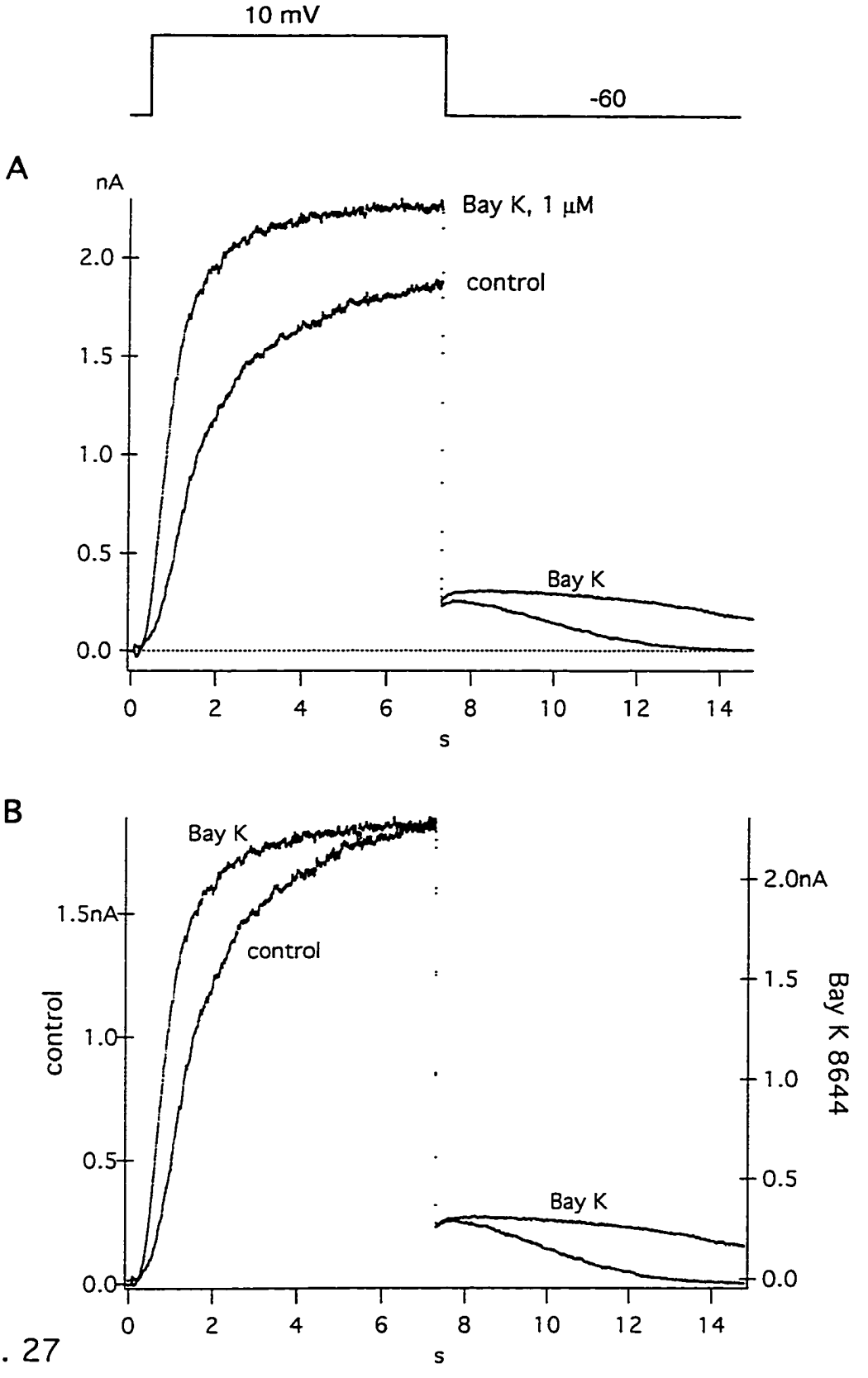


Fig. 27

could also evoke the $I_{K(Ca)}$. As shown in Fig. 28A carbachol evoked a large current which reversed at -40 mV (close to E_K in 30 mM K^+). The response desensitized rapidly. After wash of carbachol external potassium was lowered to 5 mM and carbachol applied again (Fig. 29B). This time the evoked current reversed at -85 mV, close to E_K. In the same cell, voltage steps also could evoke the $I_{K(Ca)}$.

Fig. 28C shows that the $I_{K(Ca)}$ is activated at depolarized voltages. Upon removal of external Ca^{2+} (substituted with Mg^{2+}) the $I_{K(Ca)}$ was abolished. Carbachol could activate the channels also when the external Ca^{2+} was removed. In Fig. 28D the carbachol effect in the absence of external Ca^{2+} is shown. The evoked current reverses close to the E_K and has a linear shape over the whole voltage range. This result is consistent with the interpretations that carbachol exerts its actions by releasing Ca^{2+} from the internal stores by a mechanism independent of external Ca^{2+} influx.

We next examined the carbachol-induced increase of the $I_{K(Ca)}$ under conditions where the latter was activated by a long depolarizing voltage step. In Fig. 29A carbachol (100 μM) increased the outward current activated by depolarization. The time course of activation changed, as seen by the shorter rise time. To test whether carbachol activates the same $I_{K(Ca)}$ current as the voltage step we applied ChTX (200 nM) after the wash of carbachol. Fig. 29B shows that ChTX blocked effectively the outward current and revealed a contaminating $I_{Cl(Ca)}$ current as judged from the inward tail. Subsequent

Fig. 28. Carbachol activated the slow K(Ca) current in the presence or absence of external Ca^{2+}. All panels show recordings from the same cell. **A.** Ramp in the range of -110 to +50 mV was applied to a βTC-3 cell which exhibited a K(Ca) current in normal external Ca^{2+} (2 mM) and 30 mM K^+. 50 μM carbachol activated a current which crossed the steady state ramp I-V at -40, close to the calculated E_K=-42.1 mV. **B.** Upon washout of carbachol, the external K^+ was reduced to 5 mM and 100 μM carbachol applied again. The carbachol-sensitive current reversed at around -85 mV close to E_K. Carbachol was washed off afterwards. **C.** Shown is the steady state ramp I-V in the presence and absence of external Ca^{2+}. In **D**, in the absence of external Ca^{2+}, carbachol (200 μM) still elicited a linear current which reversed at -85 mV. (The experiments are from the same cell and were performed sequentially-A, B, C, D.)

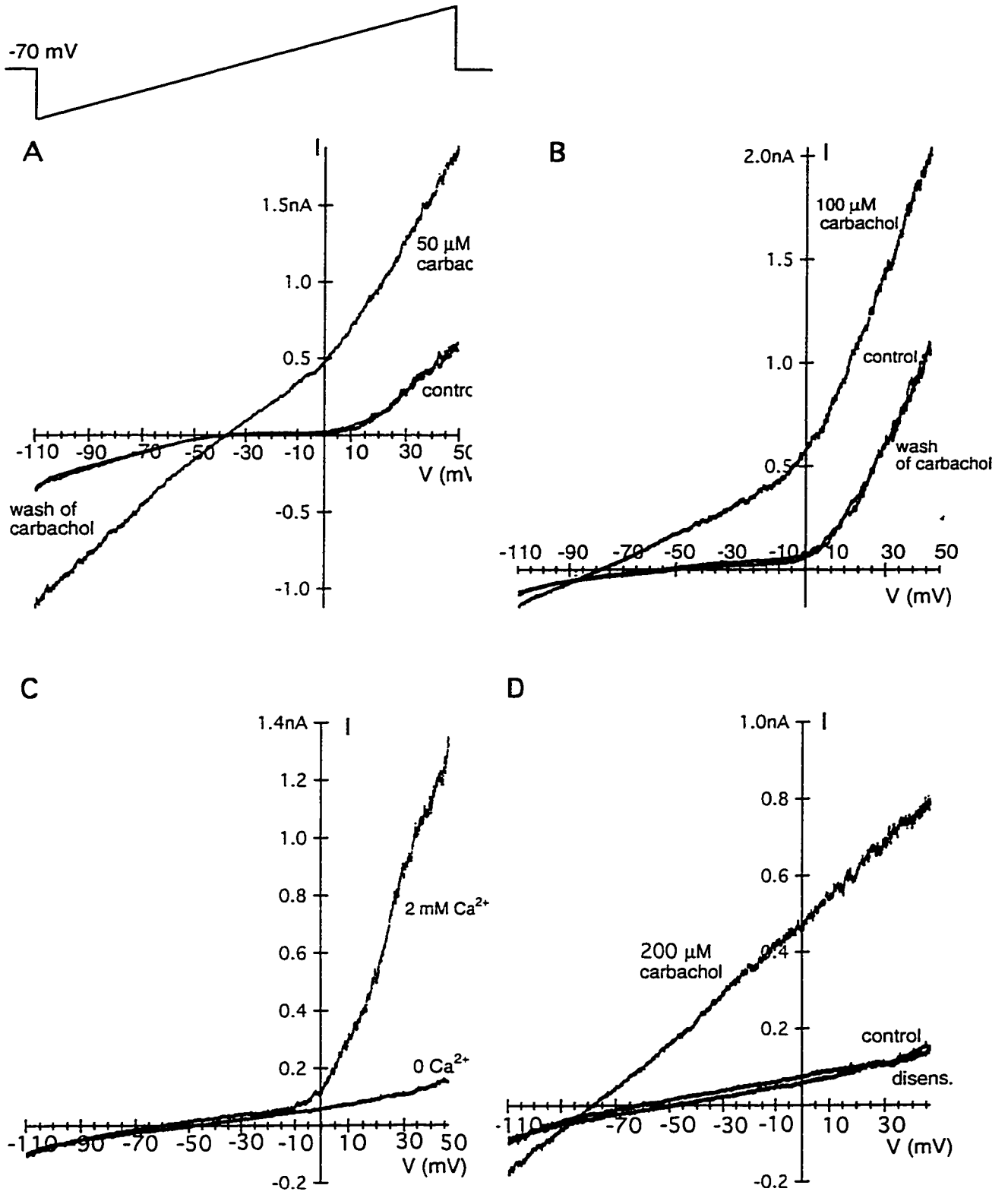


Fig. 28

application of carbachol (100 μM) in the presence of the toxin resulted in a very modest increase in the outward current, which was expected if carbachol acts on the ChTX-sensitive current (presumably the unblocked fraction). The increase in the outward current can probably be attributed to the activation of the $I_{Cl(Ca)}$, which is present in this cell as seen by the inward tail current at -60 mV. After washing carbachol and ChTX, carbachol (100 μM) was applied again (Fig. 29D) and showed a much greater effect on the outward current than in panel C. Therefore the small carbachol effect in C is most probably not due to desensitization of the muscarinic receptor.

The time course of the carbachol effect is similar to $I_{Cl(Ca)}$ activation as discussed above (Fig. 15). As with $I_{Cl(Ca)}$, carbachol and muscarine (50-200 μM) caused regular oscillations of $I_{K(Ca)}$ in most cells (n=6) although in some cells only one peak increase in current was seen. In Fig. 30, the cell was held at -40 mV, a potential at which the K^+ current should be outward and the Cl^- current-inward. At this potential the driving force for Cl^- is low, thus enabling us to examine carbachol effects on the $I_{K(Ca)}$ preferentially. The holding potential was not more depolarized as to avoid activation of the L-type Ca^{2+} channels (see Fig. 2). As seen from the inset this cell had a pronounced inward rectifier K^+ current and this we used to measure the time of arrival of the agonist at the cell surface (as described in Fig. 15). In panel A the oscillations elicited by 100μM carbachol are shown. Their frequency is similar to the frequency of $I_{Cl(Ca)}$ oscillations (Chapter 4, Fig.15).

Fig. 29. The current activated by carbachol is ChTX-sensitive. **A.** Carbachol increases the amplitude of the $I_{K(Ca)}$ elicited by a depolarizing voltage step. Note that both the "onset" and the outward tail currents are affected. **B.** After washing off carbachol, 200 nM charybdotoxin blocked most of the outward current (voltage-activated) and revealed an inward tail. It is most likely that this cell has a contaminating $I_{Cl(Ca)}$ conductance which is revealed when most of the $I_{K(Ca)}$ is blocked by ChTX. Hence the change in the direction of the tail from outward ($I_{K(Ca)}$+$I_{Cl(Ca)}$) to inward (only $I_{Cl(Ca)}$). **C.** In the presence of ChTX second application of carbachol (100 μM) causes a small increase in the outward "onset" current. **D.** After washing off carbachol and ChTX carbachol (100 μM) is applied for the third time, causing a larger increase in the outward current.

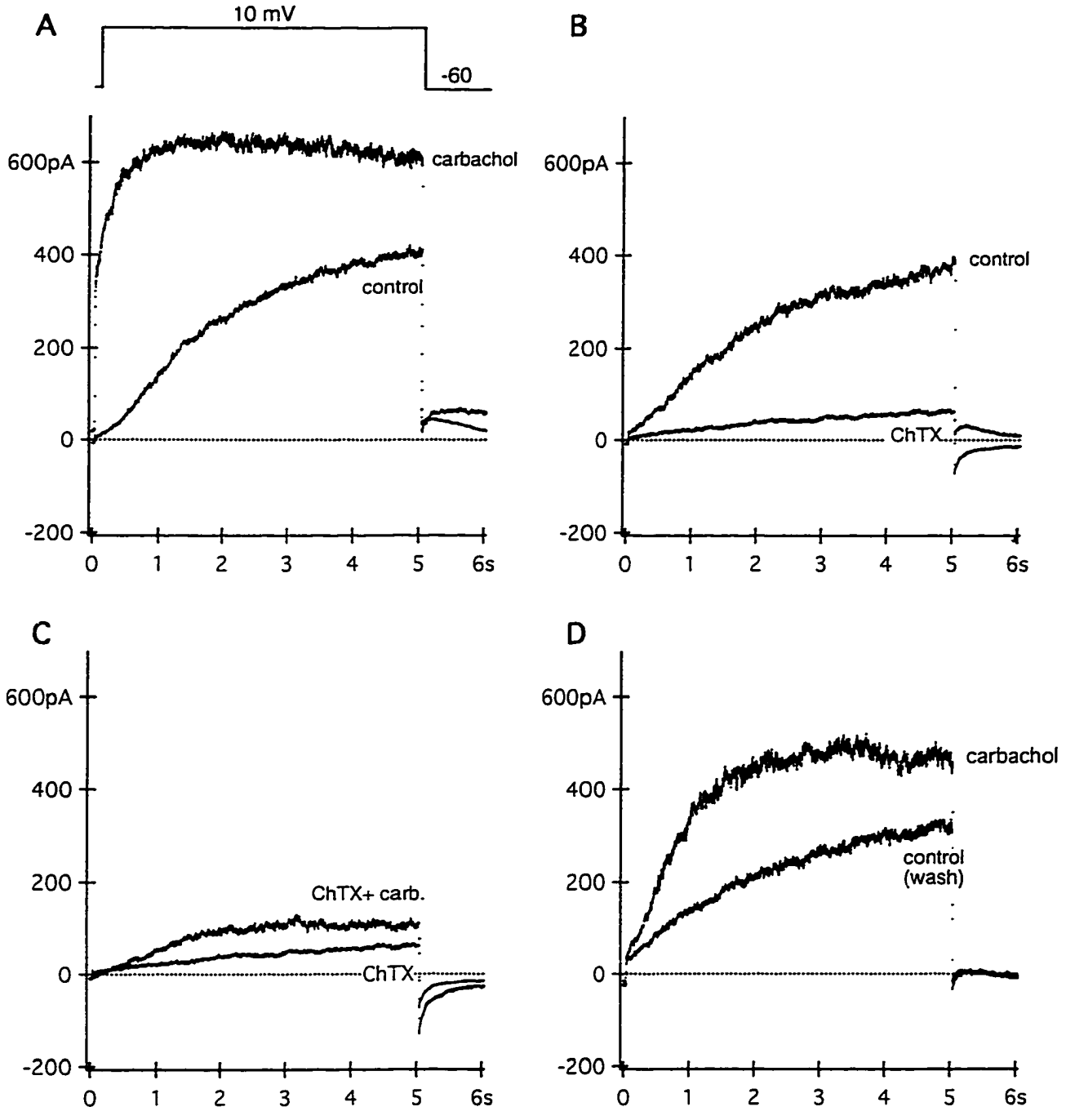


Fig. 29

Fig. 30. Time course of carbachol-induced activation of $I_{K(Ca)}$. At a holding potential of -40 mV the cell is bathed in B-30 K, 10 TEA. Carbachol is dissolved in B-5K solution. Upon the start of perfusion the low K^+ causes the inward rectifying K^+ current to diminish which is visible as an upward inflection of the current. The inset shows the I-V of the cell in B-5K external solution. Internal solution: B-I-K-sulphate.

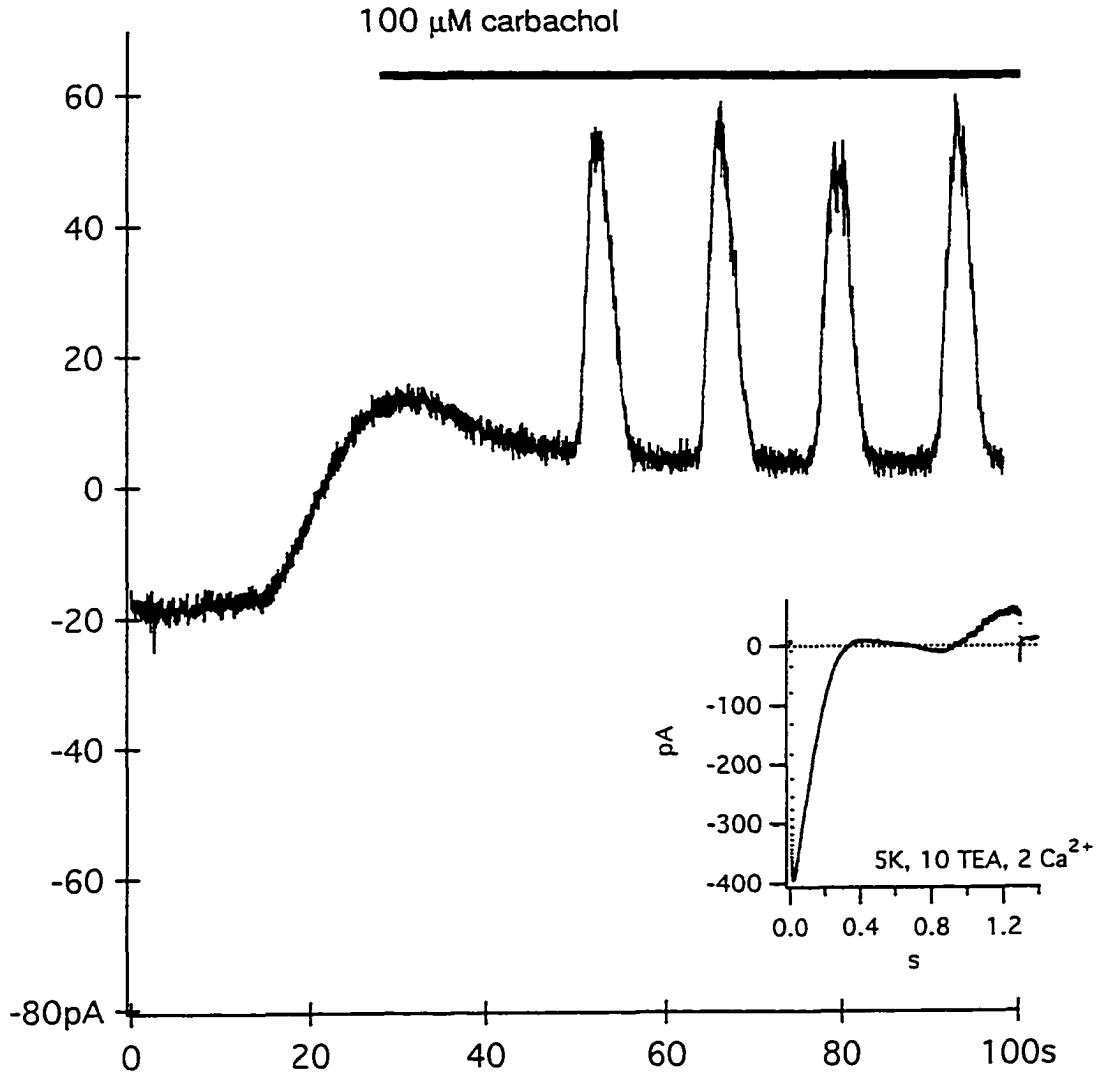


Fig. 30

Existence of a slow $I_{K(Ca)}$ in acutely isolated porcine islet cells

We next tested whether a slow $I_{K(Ca)}$ conductance appears also in acutely dissociated, "normal" β-cells. Conditions similar to those described for βTC-3 cells were used for pig islet cells. Fig. 31 shows representative recordings from isolated porcine cells. In panel A, a 6 s depolarizing step evoked a slowly-activating outward current followed by a characteristic slow tail. The current could be blocked reversibly by 100 μM Cd^{2+} like its βTC-3 analog (see Fig. 18). The inset shows the early part of the record and the I_{Ca} current block by Cd^{2+}.

The non-specific K^+ channel blocker quinidine (200 μM) completely abolished the Ca^{2+}-sensitive current (n=4) (data not shown). The K(Ca) current was seen in 16 porcine cells and was insensitive to external TEA (5 mM) (n=3) (data not shown). Pig cells consistently displayed a fast outward A-like K^+ current which was sensitive to 4-AP. In panel B a recording from a cell is shown which displayed a pronounced A-like K^+ current. First, Cd^{2+} was applied, it affected only the slow current but not the spike of the A-current. In the presence of 100 μM Cd^{2+} 6.4 mM 4-AP completely abolished the A-type current as shown in the inset. The Cd^{2+} effect was reversible. Thus, the porcine K(Ca) current appeared similar to the $I_{K(Ca)}$ in βTC-3 cells (Fig. 18) with

Fig. 31. The slow Ca^{2+}-activated current is present in acutely dissociated porcine islet cells. **A.** Long depolarizing pulses to 20 mV evoked a slowly activating outward current which was blocked by 100 μM Cd^{2+}. The inset shows the initial part of the recording where the block of the I_{Ca} is seen. **B.** A similar experiment in another porcine islet cell, which exhibited a large transient outward (A-type) current. 100 μM Cd^{2+} failed to affect the A-type current but completely abolished the slowly activating current. In the presence of Cd^{2+} 6.4 mM 4-AP blocked the transient current. The Cd^{2+} effect was reversible. The inset shows the initial part of the record and demonstrated the 4-AP effect on the transient current.

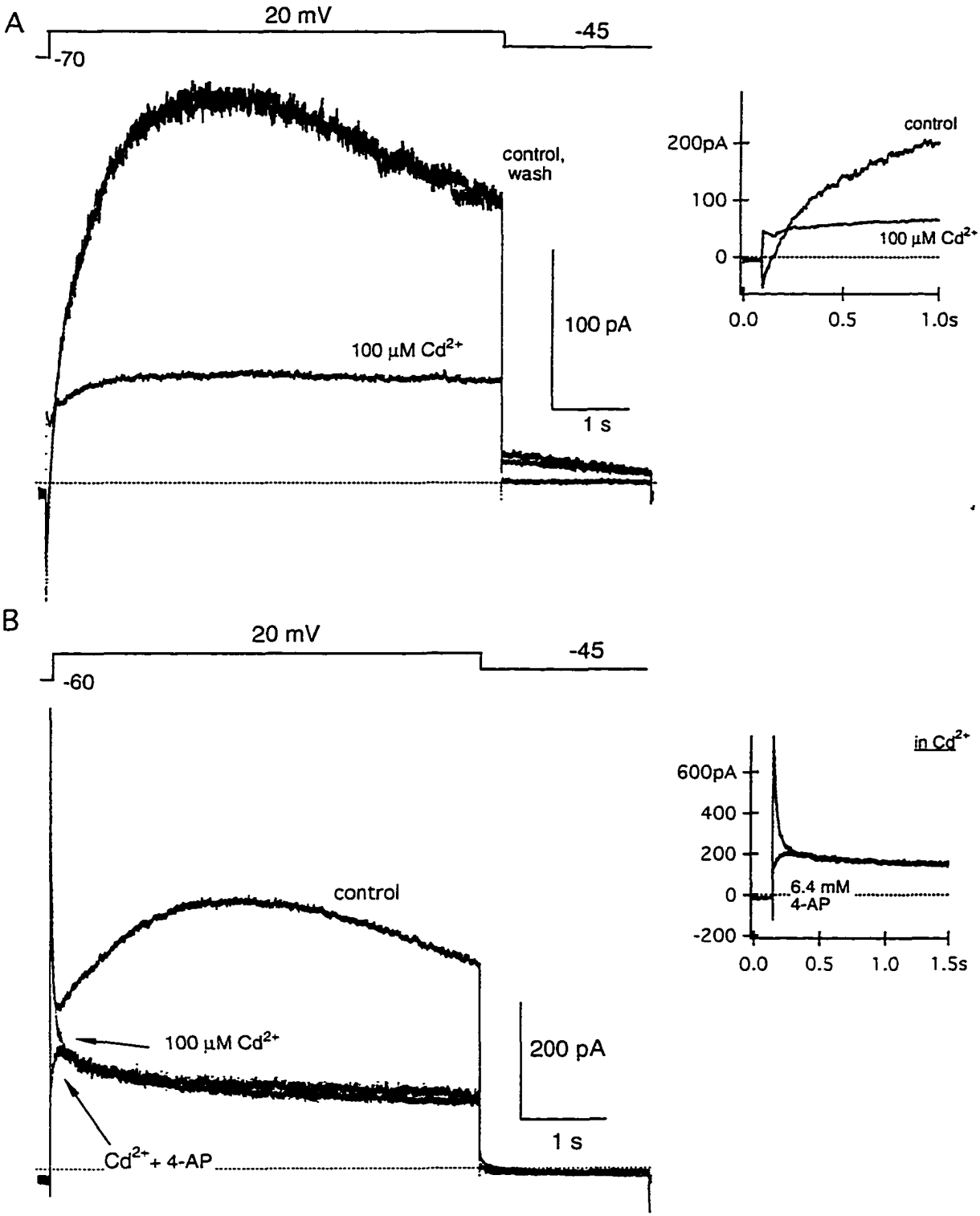


Fig. 31

respect to kinetics, TEA and quinidine sensitivity. However charybdotoxin at concentrations 100-200 nM was ineffective in blocking the current (n=5), suggesting species differences in the pharmacological characteristics of these K(Ca) currents.

Carbachol had no effects on the pig islet cell ion currents in 3 cells where it was tested.

The electrical activity of βTC-3 cells and its modulation by carbachol

βTC-3 cells occasionally exhibit regular firing patterns. We correlated the firing behavior with the presence of $I_{K(Ca)}$ in the cell under whole cell voltage clamp. The presence of the $I_{K(Ca)}$ in a cell was necessary and sufficient for the cell to exhibit firing behavior (n=10). Generally the firing was spontaneous, but in some cases current injection of 10-20 pA initiated the spiking. The cell activity in one such cell is shown in Fig. 32. From the baseline of approximately -60 mV, the cell was spiking with a frequency of around 0.2-0.3 Hz. Under whole cell voltage clamp a K(Ca) current could be evoked by depolarization. Addition of ChTX caused the repolarization phase of the spikes to become more positive. The effect of ChTX was reversible and repeatable in the same cell (not shown).

In another firing cell the effect of carbachol was examined (Fig. 33). Upon application of carbachol the firing ceased for a period of time (approximately

Fig. 32. The electrical activity of the βTC-3 cell can be modified by charybdotoxin. A βTC-3 cell spontaneously firing was bathed in B-5K solution. Charybdotoxin (100 nM) effect is shown. ChTX effect was reversible. Internal solution was B-I-K-sulphate.

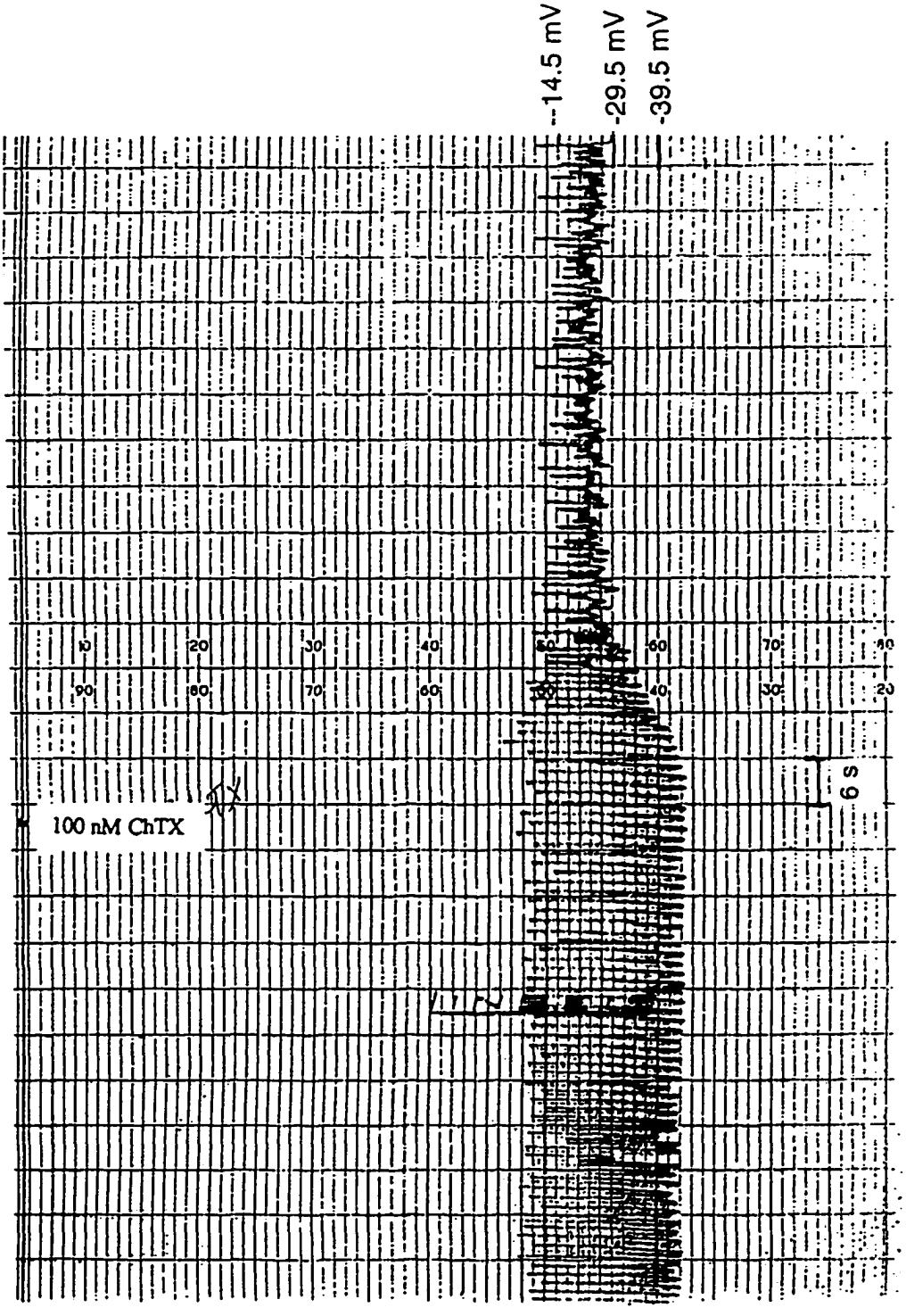


Fig.32

Fig. 33. The electrical activity of a βTC-3 cell can be modulated by carbachol. A spiking cell which had a large $I_{K(Ca)}$ when measured under voltage clamp. Application of 100 μM carbachol caused cessation of the spiking activity which subsequently resumed despite the continued presence of agonist. Internal solution: B-I-K-sulphate, external solution: B-5K. The calibrating mark shows the level of -60 mV.

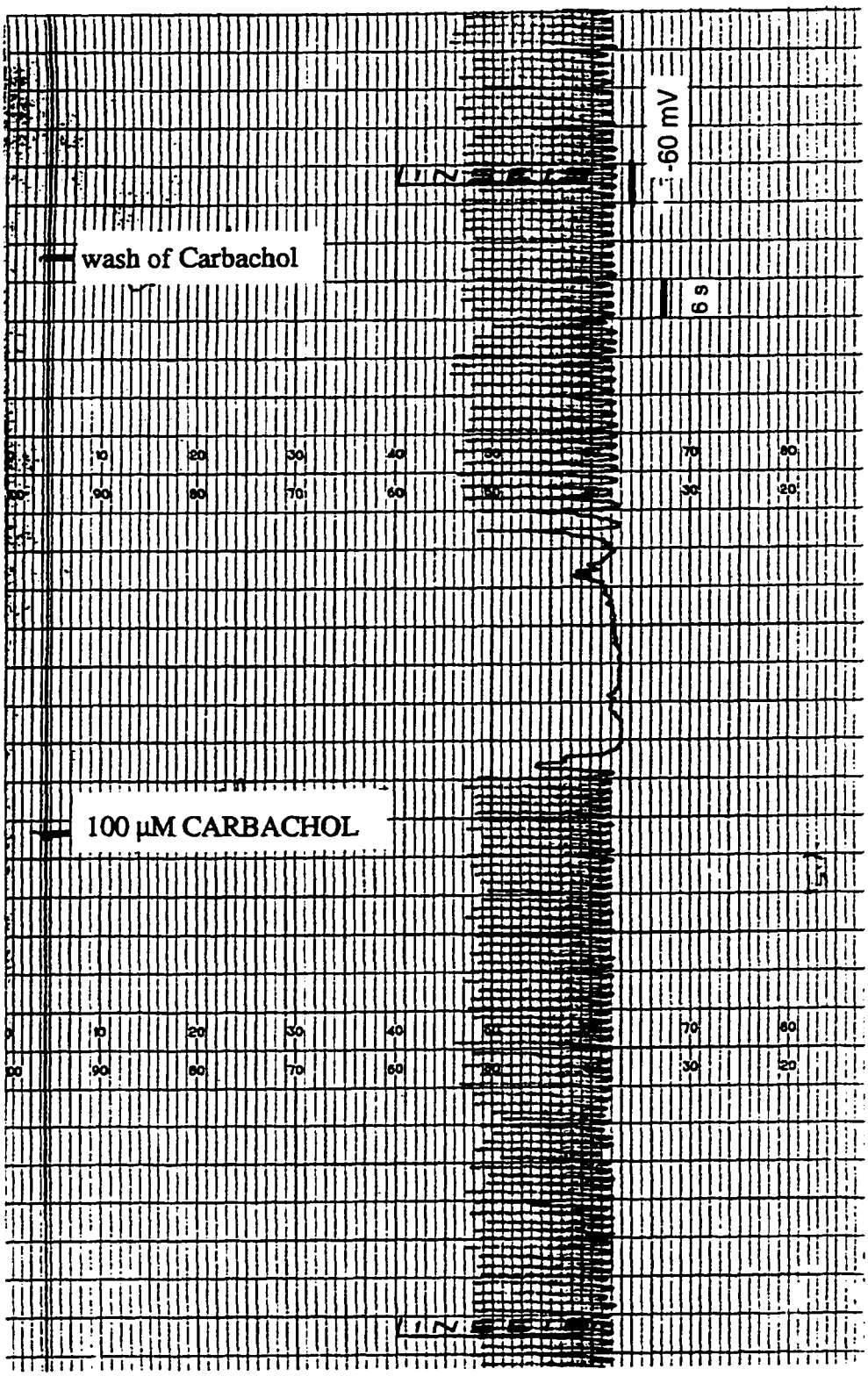


Fig. 33

35 s), after which the firing was resumed in the continued presence of carbachol (100 μM). The silent period caused by muscarinic stimulation was slightly more hyperpolarized than the baseline.

This result is consistent with the observed carbachol effects under voltage clamp, where the response desensitizes in approximately 30 seconds. The resumption of firing in the presence of carbachol most probably reflects this desensitization of the muscarinic receptors (see *Discussion*). This cell also exhibited a K(Ca) current under voltage clamp.

Chapter 6

Discussion

The calcium current in βTC-3 and pig islet cells

The depolarizing phase of the action potential in the pancreatic β-cells reflects the activation of the voltage-dependent calcium channels (VDCC) (Rorsman et al. 1994). VDCCs provide the primary path for calcium influx during an action potential and possibly during plateau potentials (Petersen & Findlay 1987; Findlay, Ashcroft, Kelly, Rorsman, Petersen & Trube 1989; Rorsman et al. 1994; Satin & Smolen 1994). Several detailed studies of calcium currents in rodent β-cells are available (Rorsman & Trube 1986; Plant 1988; Ashcroft, Kelly & Smith 1990; Smith, Ashcroft & Fewtrell 1993). Our study shows that the calcium current found in βTC-3 cells is similar to the "L-type" current described in mouse β-cells (Rorsman & Trube 1986; Plant 1988). The current activates at voltages positive to -40 mV, peaks at -10 to 0 mV and shows little time-dependent inactivation. It is blocked reversibly by micromolar concentrations of Cd^{2+}. Barium and strontium but not magnesium are able to carry the current as is the case in numerous other tissues (Hagiwara & Byerly 1981). In the absence of divalents, Na^{+} and other monovalent cations are permeant also. It is sensitive to dihydropyridine drugs nifedipine and Bay K (nanomolar) as well as to the phenylalkylamine verapamil. Mouse β-cells are thought to have only the L-type calcium conductance. In contrast, rat β-cells possess the transient T-type conductance in addition to the L-type (Ashcroft et

al. 1990). We failed to observe a T-type conductance when the holding potential was shifted to more negative values, where both L- and T-type channels are likely to be activated (Findlay et al. 1989).

The porcine Ca^{2+} current was studied in less detail. It exhibited faster inactivation at a sustained depolarizing potential (time constant around 200 ms). No evidence for the existence of a T-type channels was found in the porcine islet cells.

The $I_{Cl(Ca)}$ conductance

Our results indicate that the insulin secreting βTC-3 cells possess an outward current, $I_{Cl(Ca)}$, which can be induced by applying depolarizing steps. It is shown that the outward current is sensitive to chloride channel blockers (niflumic acid and DIDS) and shifts its reversal potential when external chloride is reduced. It is proposed that the depolarizing steps activate the $I_{Cl(Ca)}$ indirectly, that is, calcium influx, evoked by depolarization, raises cytosolic Ca^{2+}, which in turn activates the Cl^- current by binding to the cytoplasmic side of single Cl(Ca) channels (Taleb, Feltz, Bossu & Feltz 1988). Several lines of evidence that we present support this idea. Depolarization fails to elicit the $I_{Cl(Ca)}$ current when calcium is omitted from the extracellular medium or when cadmium is added. Increasing duration of the depolarizing steps elicits progressively increasing outward current and associated tail currents. This is expected because longer steps will activate "L-like" calcium currents for a

longer period of time and bring more calcium into the cell. The current does not decay with time, just as the calcium-dependent chloride currents described in neurons (Owen, Segal & Barker 1984; Mayer 1985; Owen, Segal & Barker 1986; Bader, Bertrand & Schlichter 1987), pituitary cells (Korn & Weight 1987; Korn & Horn 1989) and smooth muscle fibers (Lamb et al. 1994). The cardiac $I_{Cl(Ca)}$ by contrast, was shown to be transient, which was attributed to the calcium transient and not the indigenous inactivation of the $I_{Cl(Ca)}$ (Zygmunt & Gibbons 1992). This is also in agreement with our study since calcium influx in our case is governed by the non-inactivating calcium current (discussed above), thus giving rise to a sustained outward chloride current. It was shown in avian neurons that both transient and non-inactivating calcium currents can evoke $I_{Cl(Ca)}$ (Bader et al. 1987). It can be hypothesized that in rat β-cells, which possess the T-type calcium current, the latter could elicit a transient $I_{Cl(Ca)}$.

There are reports that barium (Miledi & Parker 1984) and strontium (10 mM) (Maricq & Korenbrot 1988) are capable of activating $I_{Cl(Ca)}$ in other systems. Although both Ba^{2+} and Sr^{2+} can carry current through the Ca^{2+} channels in our preparation, neither of them could elicit the chloride current when substituted on a mole for mole basis for calcium. Interestingly, 2 mM external Sr^{2+} activated the slow calcium-dependent potassium current present in these cells.

We also examined the selectivity of the calcium-dependent chloride current to different anions. In agreement with the results of Evans and Marty

Evans & Marty 1986 (1986), we obtained the following selectivity sequence I>NO_3>Br>Cl>Acetate, with permeability ratio 2.4>2.1.>1.7>1>0.5. This corresponds to an Eisenman I sequence and is characteristic of other types of chloride currents as well (Gray, Bevan & Ritchie 1984; Gögelein, Schlatter & Greger 1987).

What is the role of $I_{Cl(Ca)}$ in β-cell function?

Although neither the exact chloride concentration nor the precise role of $I_{Cl(Ca)}$ in βTC-3 cells are known, this current is a likely candidate for a proposed Ca^{2+}-dependent conductance regulating the bursting activity in insulin-secreting β-cells. In other tissues where $I_{Cl(Ca)}$ has been described, it has been implicated in spike after-depolarization (Mayer 1985) and after-hyperpolarization (Kom, Bolden & Horn 1991). Data obtained using gramicidin as a perforating agent (as discussed in *Methods*, this allows one to avoid alterations in intracellular Cl^- concentration), suggest that the physiological reversal potential of $I_{Cl(Ca)}$ is close to -22 mV; as discussed in *Methods*, this approach minimizes changes in intracellular Cl^- concentration. Assuming that this estimate of the Nernst equilibrium potential for Cl^- is similar to that in "normal" β-cells, activation of $I_{Cl(Ca)}$ would tend to depolarize the membrane potential of the β- cell towards E_{Cl}.

Using mouse β-cells Eddlestone & Beigelman 1983 (1983) investigated the influence of external chloride on the mouse β-cell membrane potential. In the absence of glucose, bath application of SITS, a blocker of chloride transport, caused membrane depolarization and a simultaneous increase in the input resistance. The depolarizing effects were reversed by increasing the external Ca^{2+} concentration and was prevented by the prior application of 2,4-DNP, presumably through the latter's ability to release Ca^{2+} from internal stores. These results could at least in part be explained by direct effects on the $I_{Cl(Ca)}$. Both SITS and DIDS (used in our study) are disulfonic stilbene derivatives and common chloride channel blockers (Inoue 1985), and the Ca^{2+} manipulations could clearly affect $I_{Cl(Ca)}$.

Sehlin& Meissner 1988 (1988) showed that in the presence of glucose, reduction of extracellular Cl^- (by substitution with impermeant anions) shifts the silent phase potential to more positive values and can stimulate the regular bursting pattern of activity by increasing the plateau fraction. In addition, chloride reduction had more complex effects on the membrane potential of mouse β-cells: it initially stimulated bursting electrical activity but then gradually inhibited it. The inhibition was characterized by a decreased fraction of the plateau phase and a reduced amplitude of the slow waves. Based on the assumption that the depolarization phase of the slow waves is due to Ca^{2+} influx into the cell, the authors proposed that β-cells possess an electrogenic Cl^- flux which could in turn influence the voltage-dependent Ca^{2+}

influx. The $I_{Cl(Ca)}$ described in this dissertation could play this role by serving as a feedback control curbing further calcium influx.

The currents underlying the burst activity in islet cells are unknown. Recent studies have shown that the K-ATP channels are unlikely to be responsible for the burst activity. A series of experiments performed in the presence of specific K-ATP channel blockers tolbutamide and glibenclamide showed that increases of external Ca^{2+} or increased Ca^{2+} influx by addition of ionophores induce bursting activity in β-cells. The bursts are accompanied by high frequency (2-4 min^{-1}) Ca^{2+} oscillations (Valdeolmillos, Santos, Conteras, Soria & Rosario 1989; Santos et al. 1992). Moreover, the periodically acitvated conductance was dependent on calcium influx through the L-type channels (Rosario et al. 1993). Charybdotoxin, TEA, quinine or tolbutamide failed to suppress bursts of action potentials evoked by increasing external calcium, making it unlikely that Maxi K(Ca) or K-ATP channels were involved. It is possible that $I_{Cl(Ca)}$ represents the postulated depolarizing conductance underlying the burst activity.

The effects of chloride current activation may also be coupled to the K-ATP channels. The K-ATP channels, believed to control the resting potential of the β-cell, recently were shown to be affected by the intracellular chloride ion concentration changes in a complex fashion (Takano & Ashcroft 1994).

A different Cl^- channel, volume activated, was described in insulin-secreting cells recently (Kinard & Satin 1995; Best, Sheader & Brown 1996). This current exhibits strong voltage dependence and outward rectification and

is not Ca^{2+}-sensitive. We occasionally observed this current in βTC-3 cells, it usually developed after a prolonged recording. The Ca^{2+}-dependent Cl^- current, however, has not been reported previously in β-cells. Several possibilities may account for the failure to identify $I_{Cl(Ca)}$ in other studies: the current may be of smaller size in native cells than in the βTC-3 cell line, most voltage clamp studies of dissociated β-cells have been done using whole cell patch-clamp recording instead of perforated-patch recording mode. Under abnormal Ca^{2+} buffering the current may not be visible, or it may run down quickly. Finally as stated above, $I_{Cl(Ca)}$ is apparent after prolonged voltage step protocols (>100 ms).

The Ca^{2+}-activated K^+ conductance

A Ca^{2+}-activated K^+ current has been characterized which shows slow activation and deactivation kinetics, is insensitive to apamin and distinct from other K(Ca) channels, such as the Maxi K^+ or SK channels.

It is demonstrated here that a TEA-insensitive Ca^{2+}-activated K^+ current, sensitive to block by ChTX can be activated by influx of Ca^{2+} through the L-type Ca^{2+} channels. It is also shown that the same K^+ channels can be activated by muscarinic agonists, even when the VDCCs are blocked, presumably through muscarinic release of Ca^{2+} from internal stores.

Properties of the Ca^{2+}-activated K^+ current

These data demonstrate that long depolarizing voltage steps (>100 ms) can evoke a slowly-activating outward K^+ current in physiological Ca^{2+}. Upon hyperpolarization a slow tail current is obtained. Both the onset and the tail current are abolished by removal of external Ca^{2+} , addition of Cd^{2+} or organic Ca^{2+} channel blockers. Increasingly longer depolarizing voltage steps evoke progressively larger currents. The K(Ca) current can be activated by calcium influx through the L-like Ca^{2+} channels. The L-type channel agonist Bay K 8644 increases this $I_{K(Ca)}$, while specific L-type channel antagonists nifedipine and verapamil, abolish it. The slow activation and deactivation kinetics of this apamin-insensitive K_{Ca} current are likely to be dependent on the amount of Ca^{2+} influx. Bay K (1μM), a Ca^{2+} channel agonist applied at (1μM), increased the rate of K(Ca) current activation while it decreased its rate of deactivation (the tail). These results are consistent with the interpretation that the deactivation is slowed down if more Ca^{2+} is accumulated under the membrane, since the sequestration and removal of Ca^{2+} would take a longer time.

Two divalent cations permeant through the L-type Ca^{2+} channels, Sr^{2+} and Ba^{2+}, were tested for their ability to substitute for Ca^{2+} influx in activating the $I_{K(Ca)}$. Sr^{2+} (2 mM) was capable of activating the K(Ca) current whereas Ba^{2+} (up to 20 mM) was ineffective.

The selectivity sequence of the $I_{K(Ca)}$ was examined. We found that the most permeant cation was Tl^+ followed by K^+, Rb^+ and NH_4^+. The selectivity sequence is similar to that of Maxi K(Ca) channel studied in β-cells (Tabcharani & Misler 1989). We found that Cs^+ and Na^+ were virtually impermeant through the channel. This contrasts with the apamin-sensitive small conductance K^+ channel (SK), which in other secretory cells (Park 1994) is Cs^+-permeable.

Earlier studies in β-cells have demonstrated the existence of a large conductance or Maxi K^+ channels Findlay, Dunne & Petersen 1985; Tabcharani & Misler 1989) and a K(Ca) current, shown to have small conductance by noise analysis (Ämmälä et al. 1991; Ämmälä et al. 1993). The Maxi K^+ channel, which has been studied extensively, is blocked by ChTX (20 nM) and TEA (1 mM) from the external side (Kukuljan et al. 1991). It was also shown to be sensitive to micromolar concentrations of quinine. This channel is insensitive to apamin, a bee venom toxin which is a blocker of the slow, small conductance K(Ca) channel (SK).

The small conductance K(Ca) channel could be activated by intracellular GTP and carbachol (Ämmälä et al. 1993). This current was shown to be insensitive to apamin and charybdotoxin (100 nM) (Ämmälä et al. 1991).

The $I_{K(Ca)}$ described here has unique pharmacology. It is insensitive to D-tubocurarine, apamin and scyllatoxin (leiurotoxin 1), specific blockers of small conductance (SK) K(Ca) channels found in other tissues (Burgess, Claret & Jenkinson 1981; Castle et al. 1989). Charybdotoxin, a 37 amino acid peptide

isolated from scorpion venom, has been shown to block various Ca^{2+}-dependent and -independent K^+ channels in various cell types: erythrocytes, lymphocytes, platelets and neurons (Wolff, Cecchi, Spalvins & Canessa 1988; Mahaut-Smith & Schlichter 1989; Mahaut-Smith, Rink, Collins & Sage 1990; Kunze, Bornstein, Furness, Hendriks & Stephenson 1994; see Garcia, Galvez, Garcia-Calvo, King, Vazquez & Kaczorowski 1991; Garcia, Knaus, Munujos, Slaughter & Kaczorowski 1995 for reviews), in addition to its high affinity block of the Maxi K^+ channel (Blatz & Magleby 1987; Moczydlowski, Lucchesi & Ravindran 1988). Surprisingly, ChTX at high nanomolar concentrations was found to reversibly inhibit this current (100 nM inhibits 80%). Since all our experiments were carried out in the presence of 10 mM TEA, the participation of the Maxi K^+ channel can be ruled out. Iberiotoxin (IbTX), a peptide closely related to ChTX is known to be a specific blocker of the Maxi K^+ channel at picomolar concentrations (Garcia et al. 1995). At concentrations up to 100 nM was ineffective in blocking the βTC-3 cell K_{Ca} channel. Kaliotoxin, another scorpion toxin, which blocks the neuronal Maxi K^+ channels (Crest, Jacquet, Gola, Zerrouk, Benslimane, Rochat et al. 1992) was also ineffective. Quinine (100 μM), on the other hand, completely abolished this $I_{K(Ca)}$.

Clotrimazole is an imidazole antimycotic which exerts its fungistatic action by inhibiting cytochrome P-450-dependent enzymes. Recently it was found to inhibit the erythrocyte Ca^{2+}-activated K^+ channels and smooth muscle Maxi K(Ca) channels (Brugnara et al. 1993; Rittenhouse et al. 1997; Rittenhouse, Vandorpe, Brugnara & Alper 1997. Apparently, clotrimazole and its analogs

block the Ca^{2+}-activated K^+ channels not by affecting the P-450 pathway but by binding directly to the external surface of the channel (Rittenhouse et al. 1997). We found that the βTC-3 $I_{K(Ca)}$ is blocked by clotrimazole, at low concentrations (1 μM). Neither the inward rectifier K^+ currents nor the Ca^{2+} currents are affected by this concentration of the drug (n=3, not shown). Therefore most probably, clotrimazole acts directly on the K(Ca) channel, and not by reducing Ca^{2+} influx (Hatton & Peers 1996) or by inhibiting the P-450 pathway. No definitive answer is available however.

Cl^- channel blockers niflumic, mefenamic and flufenamic acids were shown recently to potentiate Maxi K_{Ca} channels (Ottolia & Toro 1994; Gribkoff, Lum-Ragan, Boissard, Post-Munson, Meanwell, Starrett et al. 1996) and the recombinant human IsK (non Ca^{2+}-dependent) channel. We found that niflumic acid (micromolar concentrations) potently and reversibly activated the slow, apamin-insensitive $I_{K(Ca)}$ current. The niflumic acid effect was not voltage-dependent and was abolished in the presence of Cd^{2+}. In the presence of niflumic acid $I_{K(Ca)}$ did not show desensitization. $I_{Cl(Ca)}$ in βTC-3 cells is blocked by 100 μM niflumic acid (Fig. 12). Thus, niflumic acid can serve as a useful tool for distinguishing the physiological role of the $I_{K(Ca)}$ versus $I_{Cl(Ca)}$ in β-cells.

Comparing the pharmacological and biophysical properties of the βTC-3 slow K(Ca) current to the published properties of other Ca^{2+}-activated K^+ channels, it is found that it shares certain characteristics with already described channels, yet shows some unique properties. The closest channel

to the one described here seems to be the intermediate Ca^{2+}-activated K^+ channel of the erythrocyte known as the Gardos channel (Gárdos 1958; Schwarz & Passow 1983). This channel is blocked by charybdotoxin, clotrimazole and quinine but not by apamin. Channels resembling the Gardos channel are found in lymphocytes, for example (Mahaut-Smith & Schlichter 1989; Lewis & Cahalan 1995). The difference from the βTC-3 channel is that TEA at millimolar concentrations also blocks these channels. None of these channels have been cloned yet. Only recently the apamin-sensitive SK channels from brain have been cloned (Kohler, Hirschberg, Bond, Kinzie, Marrion, Maylie et al. 1996).

Muscarinic agonists activate both $I_{K(Ca)}$ and $I_{Cl(Ca)}$.

In pancreatic β-cells, acetylcholine exerts its effects through muscarinic receptors of the M3 type (Boschero, Szpak-Glasman, Carneiro, Bordin, Paul, Rojas et al. 1995), coupled to the PLC-IP_3 pathway (Malaisse 1986). IP_3 triggers large Ca^{2+} release from internal stores (Petersen, Petersen & Kasai 1994; Gromada & Dissing 1996), which in turn can act as a second messenger on various effectors. In β-cells, carbachol and other muscarinic agonists increase glucose-induced insulin release (Cook et al. 1981; Hermans, Schmeer & Henquin 1987; Gylfe 1991). The ionic basis of muscarinic action has not been elucidated (Palafox, Sanchez-Andres, Sala,

Ferrer & Soria 1985; Sherman 1996). Some studies show that muscarinic activation causes depolarization, evident as an increase in duration of plateau (Cook et al. 1981), while others have shown hyperpolarzing, evident as an increase in the interburst intervals (Henquin & Meissner 1984; Bordin et al. 1995). Ämmälä et al. (1993) showed that in single β-cells micromolar concentrations of carbachol activate the small conductance K(Ca) channel through oscillations in calcium levels. Similar oscillations of a K^+ conductance have been observed in another study (Lund & Hellman 1992 -1993). The Ämmälä et al. 1991 study gave rise to a controversy concerning the applicability of this result obtained from a cultured β-cell to the situation in an intact islet (Cook 1992). It was argued that since in normal isolated islets the muscarinic effect is stimulatory, this cannot be reconciled with the hyperpolarizing action of the K(Ca) conductance. The counter-argument brought by the authors of the study (Rorsman & Berggren 1992 was that since both depolarizing (Gagerman et al. 1978) and repolarizing (Henquin & Meissner 1984) effects of ACh had been reported, the stimulatory action in the cultured β-cells may have been lost and only the inhibitory action preserved under the culture conditions. The authors speculate that the depolarizing action may be due to a Na^+ conductance. Indeed, there is one study from Henquin's laboratory, employing Na^+ ion flux measurements in β-cell clusters which argues for the existence of such Na^+ conductance coupled to M3 muscarinic receptors (Miura, Gilon & Henquin 1996). In βTC-3 cells, however,

no effect of Na^+ ion substitutions on the muscarinic modulation of the membrane currents was seen (see also Fig. 17).

Other investigators have suggested that the depolarizing effect of muscarinic agonists may be due to a Ca^{2+}-activated Cl^- conductance (Palafox et al. 1985). Also, it has been suggested that a similar conductance may underlie the burst plateau depolarization (Pressel & Misler 1991), in analogy with the plateau phase of the action potential seen in At-t20 pituitary cells (Korn et al. 1991). These suggestions seem more probable, since the Cl^- equilibrium potential is around -22 mV, and Cl^- conductances would be depolarizing. It is harder to reconcile the value of plateau potential (≈-35 mV) with a muscarinic receptor activated Na^+ conductance, since it would drive the membrance potential to more depolarized values, towards the Na^+ equilibrium potential of ≈+60 mV.

Atwater's group (Bordin et al. 1995) demonstrated that in islets carbachol induces a Ca^{2+}-dependent K^+ conductance which is blocked partially by 50 nM ChTX. In βTC-3 cells, we show that carbachol and muscarine activate the $I_{K(Ca)}$ in the presence or absence of Cd^{2+}. This current can therefore be activated both by Ca^{2+} influx through the "L-like" channels and by Ca^{2+} release from internal stores (ER) triggered by hormonal activation. The activation of our $I_{K(Ca)}$ in the presence of TEA argues against possible current contribution by the Maxi K_{Ca} channels. The current activated by carbachol shows no bell-shaped I-V relationship seen when it is activated by voltage steps, but rather appears

to be linear. The bell shape I-V relation of $I_{K(Ca)}$ therefore is caused indirectly by the I_{Ca} I-V.

As seen in Fig. 29A, the slow time course of activation of $I_{K(Ca)}$ is changed when carbachol acts to release Ca^{2+} from internal stores. The current activates much faster, presumably because the massive release of calcium from the stores increases the Ca^{2+} concentration in the vicinity of the channels faster. This is consistent with data shown in Fig. 27, where greater amounts of calcium influx due to Bay K action cause earlier activation of $I_{K(Ca)}$.

As shown recently (Gromada & Dissing 1996), application of acetylcholine to βTC-3 cells elicits a peak rise in internal $[Ca^{2+}]_i$ caused by release from the internal stores. The internal Ca^{2+} elevation reaches maximum within 5 seconds and declines to prestimulatory levels after approximately 40 seconds. The IP_3 levels rise and fall in parallel to those of $[Ca^{2+}]_i$. Our findings of $I_{K(Ca)}$ activation by carbachol are in agreement with this study. Fig. 30 shows the time course of $I_{K(Ca)}$ activation at -30 mV which is similar to that of the $[Ca^{2+}]_i$ level fluctuations in βTC-3 cells. We also frequently observe oscillations of the current in response to muscarinic stimulation in the presence and absence of external Ca^{2+}. The lack of such oscillations in the Gromada and Dissing (1996) study may result from the concentration of ACh not being high enough. We observed oscillations when carbachol concentrations used were 100 μM or higher.

How does the βTC-3 $I_{K(Ca)}$ relate to the other K(Ca) conductances described in β-cells?

The βTC-3 $I_{K(Ca)}$ resembles the small conductance channel described in mouse β-cells (Ämmälä et al. 1991) in that it is apamin-insensitive and has slow kinetics. It is different from it by its sensitivity to ChTX. Ämmälä and colleagues tested the effects of ChTX at a concentration as high as 100 nM at various voltages (n=3). 100 nM blocked only 8% of the outward current at -40 mV, a voltage level where most of the Maxi K^+ channels are not active. TEA however in the same experiment blocked 23% of the total current. ChTX was not applied in the presence of 5 mM TEA to determine whether ChTX and TEA blocked the same current. The disparity between TEA and ChTX block (23 vs 8 %) is difficult to discern, if we assume that both agents at these high concentrations block the Maxi K^+ current alone. The evidence for ChTX insensitivity of the small conductance K_{Ca} channel needs to be clarified further. The possibility exists that the $I_{K(Ca)}$ in the βTC-3 cell line has a higher sensitivity to ChTX than its counterpart in the "normal" cells.

Kukuljan and colleagues (Kukuljan et al. 1991) showed that crude scorpion venom or application of 20 nM ChTX had no effect on β-cell bursting behavior. This finding strongly suggested that the Maxi K^+ channels are not involved in the pacemaking conductance in the bursting cells. Bordin and colleagues (Bordin et al. 1995) showed that 50 nM ChTX reduced the

carbachol-induced hyperpolarization and concluded that the Maxi K^+ channels may be involved in the muscarinic-induced hyperpolarization but not in the glucose-induced one. If the K(Ca) current described were present in mouse cells then its block by ChTX could have explained these results. It is unlikely that the Maxi K^+ channel would be involved since, its probablility of being open at negative membrane potentials is low (Tabcharani & Misler 1989). It may be the case that the $I_{K(Ca)}$ is involved in setting the interburst hyperpolarizing intervals and carbachol-induced hyperpolarizations. In a spontaneously firing βTC-3 cell, the spike repolarization becomes more negative when Bay K is applied, causing increased Ca^{2+} influx (see Fig. 3) and thus activating a larger $I_{K(Ca)}$ (not shown). While it is difficult to compare this mode of firing to the bursting activity, it is still suggestive that the hyperpolarization (silent) phase can be enhanced by increased $I_{K(Ca)}$.

The electrical activity caused by increased Ca^{2+} influx (Valdeolmillos et al. 1989; Santos, Rosario, Nadal, Garcia-Sancho, Soria & Valdeolmillos 1991; Santos et al. 1992) shows different properties depending on the mode by which the Ca^{2+} influx increased. Thus, bursting activity was reestablished when external Ca^{2+} was raised to 12.8 mM. When ionomycin (an ionophore) was applied, the membrane tended to hyperpolarize to the silent phase potential. Possibly, two underlying Ca^{2+}-dependent conductances have different sensitivity to Ca^{2+}, and therefore one of them needs much higher internal Ca^{2+} concentrations to be active. Ionomycin causes a massive Ca^{2+} influx, far greater than simply raising external $[Ca^{2+}]$. Therefore these results

imply that the K(Ca) conductance is less senisitive to Ca^{2+} than the depolarizing one (possibly the $I_{Cl(Ca)}$).

The $I_{K(Ca)}$ counterpart in pig islet cells

It was attempted to test whether the slow K(Ca) current is present in "normal", acutely isolated β-cells. In porcine dissociated islet cells, a current similar to the βTC-3 one appeared to be Cd^{2+} and quinidine-sensitive. It declines in amplitude during a prolonged depolarization, presumably because pig islet I_{Ca} inactivates faster than βTC-3 I_{Ca}. This current, however, was not sensitive to ChTX. Indeed, β-cell electrical behavior has revealed specific differences between various species. For example, in canine and human β-cells, there is a large voltage-dependent Na^+ current generating trains of action potentials which in turn activate Ca^{2+} channels (Pressel & Misler 1991; Barnett, Pressel & Misler 1995; but see also Kelly et al. 1991). In mouse and rat β-cells, on the other hand, Na^+ channels are thought not to play a significant role in the firing pattern of the cell. Differences have been noted even between rodent β-cell Ca^{2+} currents: rat and Hit-t15 (hamster insulinoma) cells are thought to posses both L and T type VDCCs, whereas mouse cells are believed to only possess slow L-type channels (Ashcroft& Rorsman 1991; Marchetti, Amico, Podesta & Robello 1994). In human erythrocytes, IbTX

inhibits the Ca^{2+}-activated K^+ channels (Gardos channels) whereas it is ineffective in rabbit erythrocytes.

Generally, single β-cells do not exhibit bursting (Rorsman et al. 1994). In our hands βTC-3 cells fire regular action potentials (Figs. 32, 33). Interestingly, we find that the presence of the $I_{K(Ca)}$ in these cells is necessary and sufficient for their firing (n=10). Thus $I_{K(Ca)}$ may have a role of a feedback conductance which can restart the firing after the plateau depolarization.

Chapter 7

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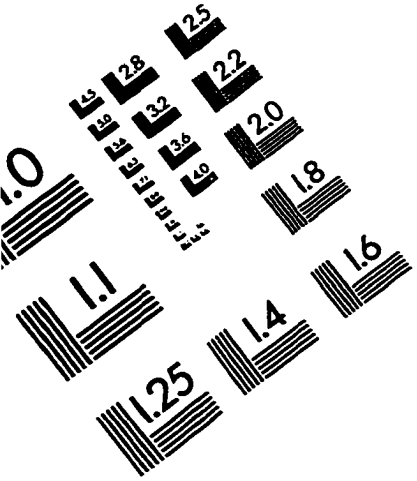
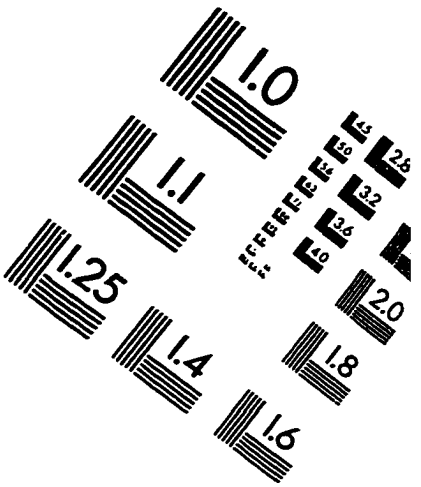
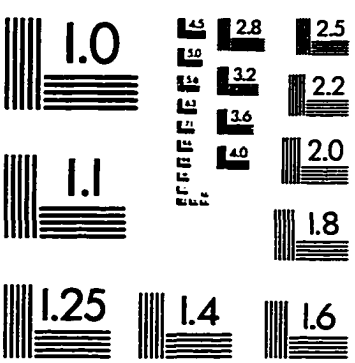
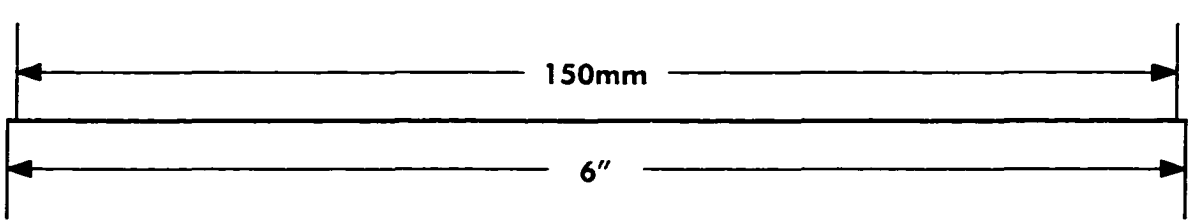
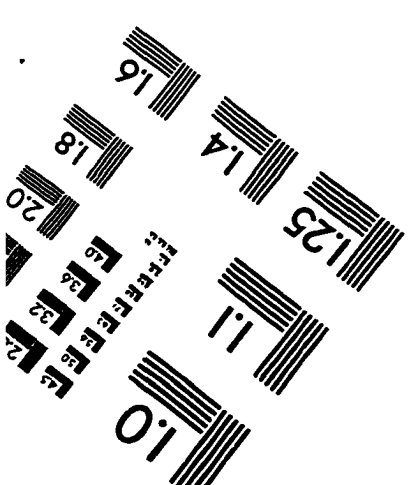
IMAGE EVALUATION TEST TARGET (QA-3)

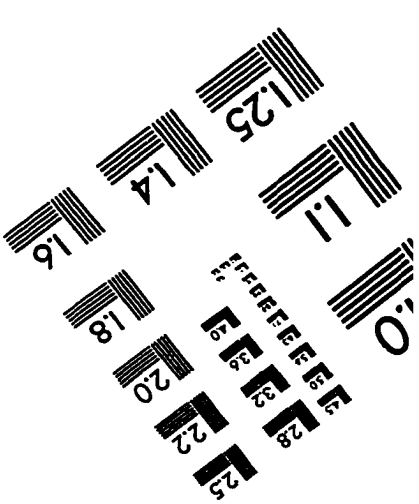












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