Journal of Physiology (1991), **443**, *pp.* 703–725
With 12 figures
Printed in Great Britain

STIMULATION-INDUCED POTENTIATION OF T-TYPE Ca^{2+} CHANNEL CURRENTS IN MYOCYTES FROM GUINEA-PIG CORONARY ARTERY

BY V. YA. GANITKEVICH AND G. ISENBERG

From the Department of Physiology, University of Cologne, Robert-Koch-Strasse 39, 5000 Köln 41, Germany

(*Received 18 February 1991*)

SUMMARY

1. Whole-cell Ca^{2+} channel currents were studied in myocytes isolated from guinea-pig circumflex coronary artery at 36 °C and with 10 mM-Ba^{2+} (or Ca^{2+}) as charge carrier. With 180 ms clamp steps from the holding potential of −100 mV, currents at −30 mV were carried mostly through the T-type calcium channels while at positive potentials currents were mostly of the L-type.

2. The increase in frequency of pulsing from 0·1 to 2·5 Hz resulted in a reduction of peak inward current ('negative staircase') with the 180 ms pulses to +10 mV, but in a 2-fold potentiation ('positive staircase') with pulses to −30 mV. T-type currents and their frequency-mediated potentiation did not change significantly when Ba^{2+} was substituted by Ca^{2+} or Sr^{2+}.

3. Potentiation of T-type currents was further analysed with a paired-pulse protocol: at a basal frequency of 0·1 Hz, a pre-pulse (inducing current I_1) was followed by a 200 ms repolarization to −100 mV and a test pulse (inducing current I_2). The potentiation could only be recorded using test pulses depolarizing the membrane to potentials between −40 and −10 mV; at more positive test potentials it was masked by the depressant effect of pre-pulses on the L-type current.

4. Potentiation of I_2 by 200 ms pre-pulses started at pre-pulse potentials more positive than −60 mV and saturated at −20 mV (I_2 potentiated by a factor 2·4). Between −20 and +130 mV the potentiation was not dependent on the pre-pulse potential suggesting that the influx of Ba^{2+} or Ca^{2+} is not required for this effect. Potentiation of I_2 by a 10 s pre-pulse followed the voltage dependence of the steady-state inactivation curve of the T-type Ca^{2+} channel; potentiation became visible at potentials more positive than −80 mV and saturated at about −50 mV.

5. When changing the interval between two identical 200 ms pulses, the T-type current was found to recover completely from inactivation within 40 ms at −100 mV; at intervals of 160–320 ms maximal potentiation of I_2 occurred.

6. With pre-pulses shorter than 200 ms, potentiation became attenuated when inactivation became less complete. When the potential during the interval between the pulses was −80 instead of −100 mV, maximal potentiation was reduced (I_2 potentiated by a factor of 1·3 instead of 2·2) and occurred later (1·28 s).

7. Potentiated T-type currents inactivated faster. In the double-pulse experiments when peak ratio (I_2/I_1) was 1·98±0·17, the current during the second pulse showed

a single exponential time course of inactivation with a time constant of $5{\cdot}6 \pm 0{\cdot}7$ ms, while the current during the first pulse had a time constant of inactivation of $14{\cdot}5 \pm 1{\cdot}5$ ms. Due to faster inactivation the second current (I_2) transported nearly the same charge compared to first current (I_1) (charge ratio $0{\cdot}94 \pm 0{\cdot}16$). Unlike the current amplitude, the transported charge did not exhibit a transient potentiation when the pulse interval was increased.

8. At -100 mV, T-type Ca^{2+} channels recovered faster from steady-state inactivation than L-type Ca^{2+} channels.

9. It is concluded from the present experiments that stimulation-mediated potentiation of T-type calcium channels is a potential-dependent process. Potentiation of T-type Ca^{2+} channels is discussed in the context of single-channel data from the literature. It is suggested that the channel passes transiently during recovery from inactivation through a closed state from which it can be open with a first latency shorter than the first latency in the steady state. Potentiation of peak current can be attributed to a larger number of channel openings within a shorter period of time.

INTRODUCTION

Although T-type Ca^{2+} channel currents have been demonstrated to exist in a variety of vascular smooth muscle cells (ear artery: Benham, Hess & Tsien, 1987; saphenous vein: Yatani, Seidel, Allen & Brown, 1987; portal vein: Loirand, Mironneau, Mironneau & Pacaud, 1989; cultured cells from aorta: Akaike, Kanaide, Kuga, Nakamura, Sadoshima & Tomoike, 1989; rat tail artery: Wang, Karpinski & Pang, 1989; coronary artery: Ganitkevich & Isenberg, 1990*a*), their physiological significance is not clear at present. The steady-state inactivation properties (Akaike *et al.* 1989) suggest that T-type Ca^{2+} channels are available only at potentials negative to -60 mV; hence, in vascular myocytes with resting potentials between -40 and -60 mV, T-type Ca^{2+} channels are thought to be permanently in the inactivated state. However, the T-type Ca^{2+} channel could recover from inactivation during the hyperpolarization evoked by neurotransmitters.

An example of physiological significance of T-type Ca^{2+} channel currents is their contribution to the pacemaker depolarization of cardiac sino-atrial cells (Hagiwara, Irisawa & Kameyama, 1988). The importance of T-type channels for Ca^{2+} influx, however, has been questioned, since in cardiac Purkinje and ventricular myocytes T-type channels recovered from inactivation more slowly than L-type Ca^{2+} channels (Hirano, Fozzard & January, 1989*a*; Tseng & Boyden, 1989). In vascular myocytes (culture from rat aorta, Akaike *et al.* 1989) recovery was faster than in other preparations. This is the reason why we studied T-type Ca^{2+} currents and their recovery from inactivation in smooth muscle cells isolated from the guinea-pig coronary artery. The experiments were performed at 36 °C and demonstrated that recovery was complete in less than 100 ms. More importantly, they show that recovery leads to a long (up to 5 s) period of potentiation during which the T-type Ca^{2+} currents have a larger amplitude and a faster inactivation than in the steady state. Some of the results have been published in abstract form (Ganitkevich & Isenberg, 1990*b*).

METHODS

Cell preparation

Single smooth muscle cells were isolated from the circumflex coronary artery of guinea-pig hearts as described earlier (Ganitkevich & Isenberg 1990*a*). Briefly, guinea-pigs were killed by cervical dislocation, and the heart was quickly removed and placed in nominally Ca^{2+}-free bath solution. The circumflex coronary artery was carefully dissected from the connective tissue and washed in nominally Ca^{2+}-free bath solution for 1 h. Then, the artery was subjected to two consecutive treatments (30–45 min each) with 1 ml of nominally Ca^{2+}-free solution containing 0·1 % collagenase (Serva, Germany) and 10 U/ml elastase (Sigma, USA). After the second treatment, the artery was transferred to nominally Ca^{2+}-free solution, and the cells were dissociated by gentle agitation with a wide-bore pipette. Cells were stored in nominally Ca^{2+}-free solution and were used on the day of preparation.

Solutions

Extracellular solutions. Ca^{2+}-free solution for the cell preparation contained (mM): 150 NaCl, 5·4 KCl, 1·2 $MgCl_2$, 20 glucose, 5 HEPES, adjusted with NaOH to pH 7·3. Extracellular solution used in most experiments contained (in mM): 110 NaCl, 20 CsCl, 10 $BaCl_2$ (or 10 $CaCl_2$) 10 HEPES, adjusted with NaOH to pH 7·3. Since the amplitude of T-type Ca^{2+} channel currents was often 20 pA or less, some experiments were done with increased extracellular Ba^{2+} or Ca^{2+} concentrations; these changes are indicated in the text. Some experiments were done with an extracellular solution containing 11 mM-$BaCl_2$.

Intracellular solutions. The patch pipettes were filled with (in mM): 130 CsCl, 2 Na_2ATP, 4 $MgCl_2$, 0·4 EGTA, 10 HEPES, adjusted with NaOH to pH 7·2. In some experiments, 130 mM-CsCl was substituted by 130 mM-NaCl. In one series of experiments (Fig. 12), L-type Ca^{2+} channel currents were suppressed by an intracellular solution which was free of ATP, $MgCl_2$ and EGTA; this solution was not generally used because it was difficult to obtain stable current recordings.

Experimental procedure

Experiments were performed at 36 °C. Initially, the same experimental set-up as in the previous work (Ganitkevich & Isenberg, 1990*a*) was used. The pulse protocols were generated by a PDP-23 microcomputer (Digital Equipment Corp., Marlboro, USA) and the currents were recorded with a List EPC-7 amplifier. Later, an IBM-compatible microcomputer and a CED-1401 interface (Cambridge Instruments, UK) were used in combination with a RK-300 patch amplifier (Biologic, France). The currents were not corrected for leakage since the input resistance was normally more than 10 GΩ. The current signal was filtered at 1 kHz and sampled with 5 kHz. Where appropriate, results are presented as means ± S.D. of the mean.

RESULTS

Smooth muscle cells from the large epicardial coronary arteries of the guinea-pig contain both T- and L-type Ca^{2+} channels (Ganitkevich & Isenberg, 1990*a*). With Ca^{2+} ions as the charge carriers, both types of inward current inactivated rapidly and were difficult to distinguish. Substitution of extracellular Ca^{2+} by Ba^{2+} slowed down the inactivation time course of L-type Ba^{2+} current but not the inactivation of T-type currents. Therefore, most experiments in this study were performed with 10 mM-$BaCl_2$ in the bath. With 10 mM-Ba^{2+} as the charge carrier, clamp steps from a holding potential of −100 mV to −30 mV induced a rapidly inactivating inward current which flowed mostly through T-type Ca^{2+} channels, whereas steps to 0 mV induced a more slowly inactivating current that was assumed to be mostly of the L-type.

Increase in pulsing frequency changed T- and L-type currents differentially

During the depolarizing clamp step Ca^{2+} channels pass through the open to the inactivated state. After repolarization, the channels return to the closed resting state. During this recovery period they become available again for activation. When

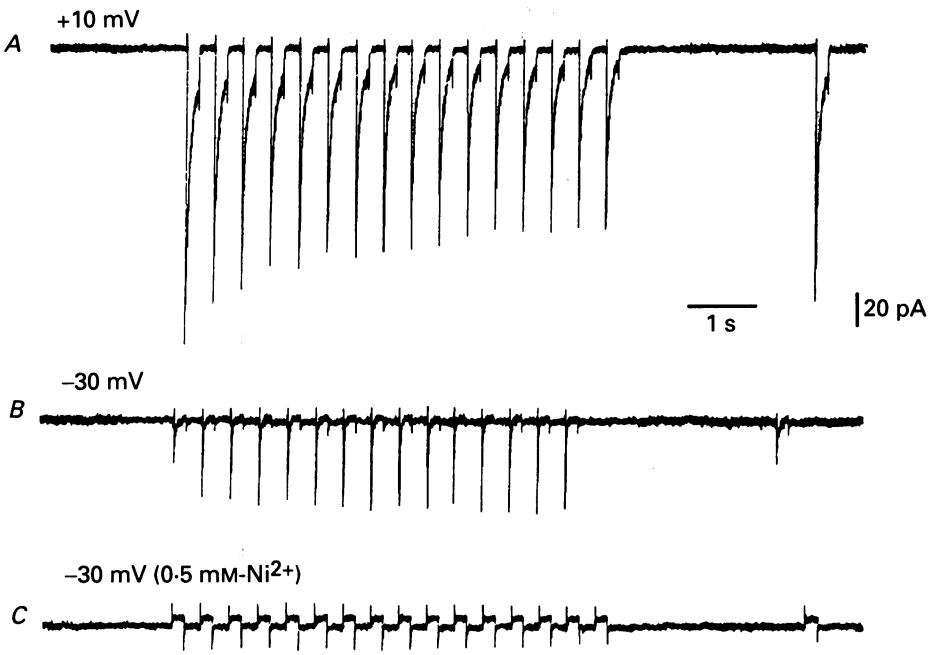

Fig. 1. Ba^{2+} inward currents at +10 mV (*A*) and −30 mV (*B*) recorded during a change in pulse frequency from 0·1 to 2·5 Hz. The last inward current shows the partial recovery of the frequency effect after a 3 s interval. *C*, currents at −30 mV after addition of 0·5 mM-$NiCl_2$ to the bath. Holding potential −90 mV, on-line pen recording. All records were from the same cell. Note: the inward current at +10 mV was depressed; the one at −30 mV was facilitated by the higher frequency.

inactivation is incomplete at the time of repolarization, some channels 'deactivate' from the open into the closed state. Recovery from inactivation takes time, and it is thought that this process limits the maximum frequency at which the current can be evoked without reduction in the peak amplitude. Figure 1*A* shows the effects of an increase in the frequency of pulsing from 0·1 to 2·5 Hz for currents (mainly of the L-type) induced by 180 ms pulses to +10 mV. During the train of pulses the peak current progressively declined ('negative staircase'). The result suggests that the 220 ms interval between the pulses was too short for the complete recovery of the channel from the inactivated to the available state. The frequency effect on the L-type current was reversible, i.e. after a 3 s interval the peak current had largely recovered.

Figure 1*B* shows the effect of 2·5 Hz pulsing on the T-type Ba^{2+} current evoked at −30 mV. In contrast to L-type Ba^{2+} current, the amplitude of the T-type current

was facilitated (potentiated), i.e. the peak amplitude increased by a factor of about 2 ('positive staircase'). After a 3 s interval, the effect had disappeared. Bath application of 0·5 mM-$NiCl_2$ abolished both the T-type Ba^{2+} current and its frequency-meditated potentiation (Fig. 1*C*). The Ni^{2+} block suggests that the net

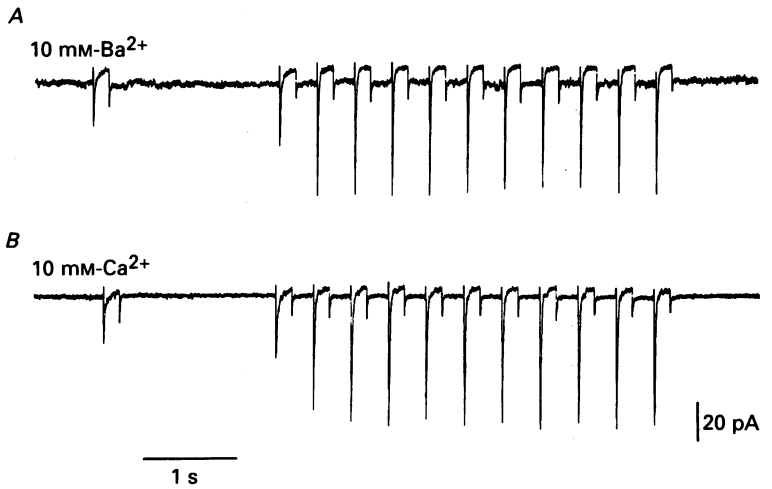

Fig. 2. Frequency-mediated potentiation of peak inward current is not dependent on the charge carrier. *A*, 10 mM $[Ba^{2+}]_o$, holding potential −110 mV, 180 ms pulses to −30 mV. *B*, 10 mM $[Ca^{2+}]_o$, holding potential −100 mV, 180 ms steps to −20 mV. Note: with Ca^{2+} as charge carrier, the potentials were set +10 mV more positive to account for the stronger screening of surface charges.

inward current is a reasonable measure of the T-type Ca^{2+} channel current at −30 mV. It further suggests that activation or suppression of other, Ni^{2+}-insensitive conductances did not significantly contribute to the observed potentiation.

The frequency-mediated facilitation of T-type current was recorded not only with Ba^{2+} but also with Ca^{2+} or Sr^{2+} ions as charge carriers. Figure 2 compares the effect of the increase in pulsing rate in the presence of 10 mM-external Ba^{2+} (panel *A*) or 10 mM-external Ca^{2+} (panel *B*) and shows that the extent of potentiation was the same. The comparison also shows that the substitution of Ba^{2+} by Ca^{2+} changed neither the peak amplitude nor the inactivation time course. The result is expected for vascular T-type Ca^{2+} channels (Akaike *et al.* 1989) but not for L-type Ca^{2+} channels, supporting the idea that the two components were reasonably separated.

Potentiation of T-type current with conditioning pre-pulses

For a more convenient evaluation, the frequency-mediated potentiation of T-type Ca^{2+} channel currents was studied with a paired-pulse protocol (Fig. 3). The paired pulses were applied at a basal rate of 0·1 Hz. Figure 3*A* shows the potentiation of the T-type Ba^{2+} current by the conditioning pre-pulse. It also shows that removal of $BaCl_2$ from the bath solution abolished both inward currents suggesting that

channels impermeable to divalent cations did not contribute to the potentiation effect.

In general, two approaches were used in the present study. Firstly, two pulses (pre-pulse, evoking current I_1 and test pulse, evoking current I_2) separated by a 200 ms

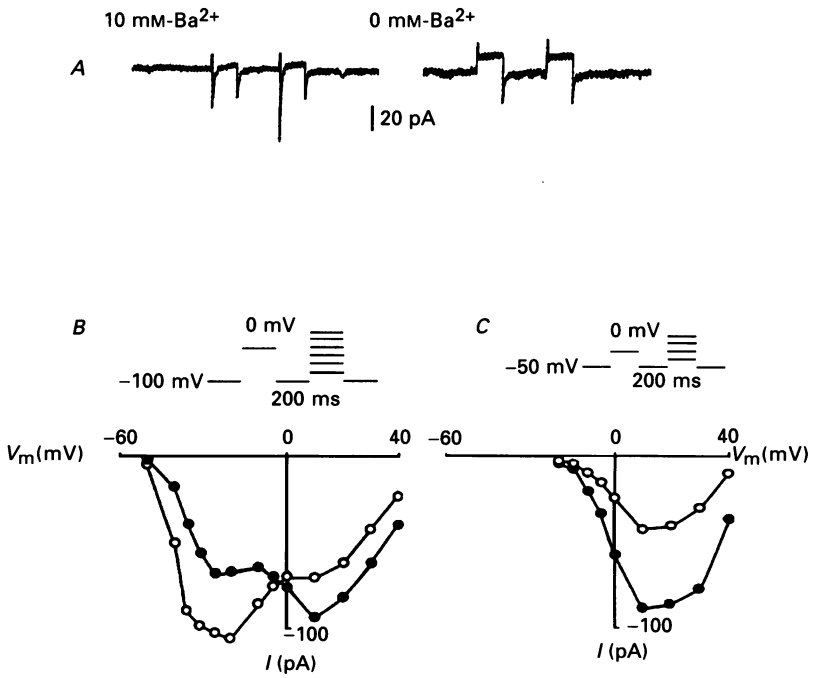

Fig. 3. Modification of the peak of the Ca^{2+} current by pre-pulses. *A*, traces (pen recording) showing currents evoked by two identical 180 ms pulses from −100 mV to −20 mV separated by 200 ms interval with 10 mM-Ba^{2+} extracellularly (left) and after Ba^{2+} ions were removed from extracellular solution (right). Recordings from the same cell. *B*, *I–V* curve in control (●) and *I–V* curve obtained with 200 ms pre-pulse to 0 mV (○, scheme is shown in the inset). Holding potential −100 mV. *C*, the holding potential was reduced to −50 mV. The control *I–V* curve (●) is shown together with the *I–V* curve obtained using 200 ms pre-pulse to 0 mV (○, scheme is shown in the inset). *B* and *C* from the same cell bathed in 10 mM-Ca^{2+}. Note: at −50 mV, T-type Ca^{2+} channels were inactivated, so they did not contribute to the peak currents plotted in the *I–V* relationship.

repolarization to the holding potential were used for characterization of the potential dependence of potentiation. Secondly, by varying the interval between two identical pulses (pulse interval) the time dependence of potentiation was studied.

Properties of potentiated T-type current

For characterization of the potentiated current I_2, the potential of the (second) test pulse was varied between −50 and +40 mV. The results were obtained in the presence of 10 mM-external Ca^{2+} and peak I_2 was plotted against the test pulse potential (*I–V* relationship, Fig. 3*B*). The control *I–V* relationship (●) was measured

without pre-pulses. It showed the contribution of T-type Ca^{2+} current as a hump of negative current between −40 and −10 mV. When 200 ms long pre-pulses to 0 mV preceded the test pulses (○), the peak test currents obtained in the potential range between −40 and −10 mV were substantially increased. At more positive potentials,

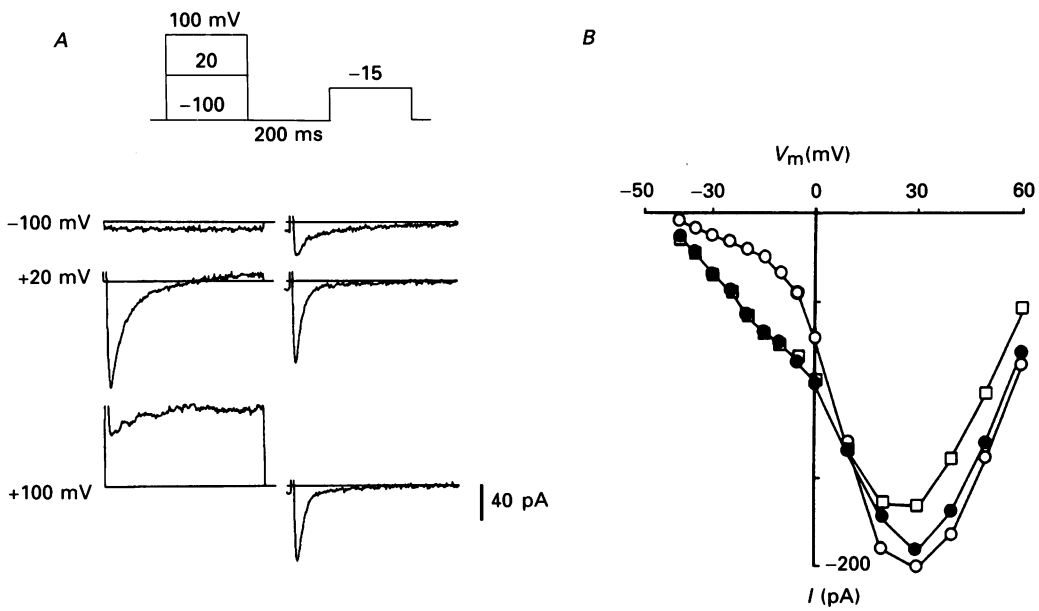

Fig. 4. Potentiation does not require Ca^{2+} or Ba^{2+} influx during the pre-pulse. *A*, 20 mM [Ca^{2+}] extracellularly. The scheme of the voltage protocol is shown in the inset. The holding potential was −100 mV. Tracings were obtained by pulse pairs consisting of 200 ms test pulses to −15 mV, and pre-pulses to −100, +20 or +100 mV as indicated at each trace. The pulses were separated by a 200 ms period at the holding potential. *B*, 30 mM [Ba^{2+}] extracellularly. *I–V* relationships of peak Ba^{2+} current (protocol similar to the one shown in Fig. 3*B*) obtained with 200 ms pre-pulses to −100 (○), +20 (●) and +90 mV (□).

however, the peak of the test current (I_2) was smaller after the pre-pulse, as can be expected from a predominantly L-type Ca^{2+} current which recovered incompletely from inactivation. The idea that the pre-pulses suppressed the L-type current is further supported by Fig. 3*C* which shows the result of the similar paired-pulse experiment but using a holding potential of −50 mV, known to inactivate T-type Ca^{2+} channels. Again, this result demonstrates that pre-pulses facilitated the T-type but not the L-type current.

For the cardiac L-type Ca^{2+} currents, a frequency-mediated increase of I_{Ca} was abolished by bath application of caffeine which is thought to deprive the intracellular stores of releasable Ca^{2+} (Tseng, 1988; Zygmunt & Maylie, 1990). In the present study 10 mM-caffeine had no effect on the potentiation of the T-type Ca^{2+} current (not shown).

To test whether other conductances could contribute to the pre-pulse potentiation of the inward current, the Cl^- concentration in the intracellular solution was reduced from 140 to 10 mM (caesium glutamate substitution) which had no visible effect on the potentiation of the peak inward current by enhanced pulse frequency or by paired pulsing. The pre-pulse-mediated potentiation persisted when 110 mM-$BaCl_2$ was used in the extracellular medium, when the intracellular medium contained 130 mM-NaCl instead of CsCl or when its pH was varied between 6·0 and 9·0.

Potentiation of the T-type current was not dependent on Ca^{2+} or Ba^{2+} influx during the pre-pulse

Potentiation of T-type current might be mediated by the influx and accumulation of divalent cations in the cell. This possibility was excluded by the results of a series of experiments using an intracellular solution in which the EGTA concentration was increased from 0·4 to 40 mM and which also contained 10 mM-BAPTA bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid). After this solution had been perfused into the cell for 3–5 min, the potentiation of the T-type currents was essentially unchanged, and neither the potentiation by increased pulsing frequency nor the one due to pre-pulses was attenuated. Also, the control Ca^{2+} or Ba^{2+} currents were not modified, as expected from a $[Ca^{2+}]_i$-insensitive T-type Ca^{2+} channel.

In a second series of experiments, the amount of Ca^{2+} or Ba^{2+} influx was modified. Figure 4*A* shows the result obtained in the presence of 20 mM-external Ca^{2+} using test steps to −15 mV. A comparison of the current traces without and with pre-pulse suggested that the peak of the T-type current was more than doubled by the pre-pulse. The factor of potentiation was 2·2 after the pre-pulse to +20 mV that evoked a large L-type Ca^{2+} current. It was similar (2·15) after the pre-pulse to +100 mV where an outward current was recorded and Ca^{2+} influx was negligible. Thus, the potentiation of the T-type Ca^{2+} current did not depend on the amount of preceding Ca^{2+} influx.

The results of Fig. 4*B* also supported this conclusion. Figure 4*B* shows *I–V* relationships measured in the presence of 30 mM-external Ba^{2+}. One curve was obtained without the application of pre-pulses (○); the other two were obtained using pre-pulses to +20 mV (●) and +90 mV (□) respectively. The comparison shows that the pre-pulses augmented the peak inward current measured in the range between −40 and +10 mV, where the contribution of T-type current was largest. In this range of potentials, the extent of potentiation by the pre-pulses to +20 and +90 was undistinguishable, i.e. it was unrelated to inflow of divalent cations.

The dependence of the degree of T-type current potentiation on the potential of a 200 ms pre-pulse is shown in Fig. 5*A*; the results from seven cells studied in 10 mM-external Ba^{2+} are presented as mean values. Potentiation by 200 ms pre-pulses started at −50 mV and increased gradually up to −20 mV; for more positive potentials the extent of potentiation remained constant. The results suggest that the potentiating effect of pre-pulses reflects a voltage-dependent gating property of the T-type Ca^{2+} channel.

Potentiation of T-type current was dependent on the extent of inactivation during the pre-pulse

The 200 ms pre-pulses, used for potentiation of I_2, activated but also inactivated the T-type Ca^{2+} channel. The rate of inactivation decreased with more negative

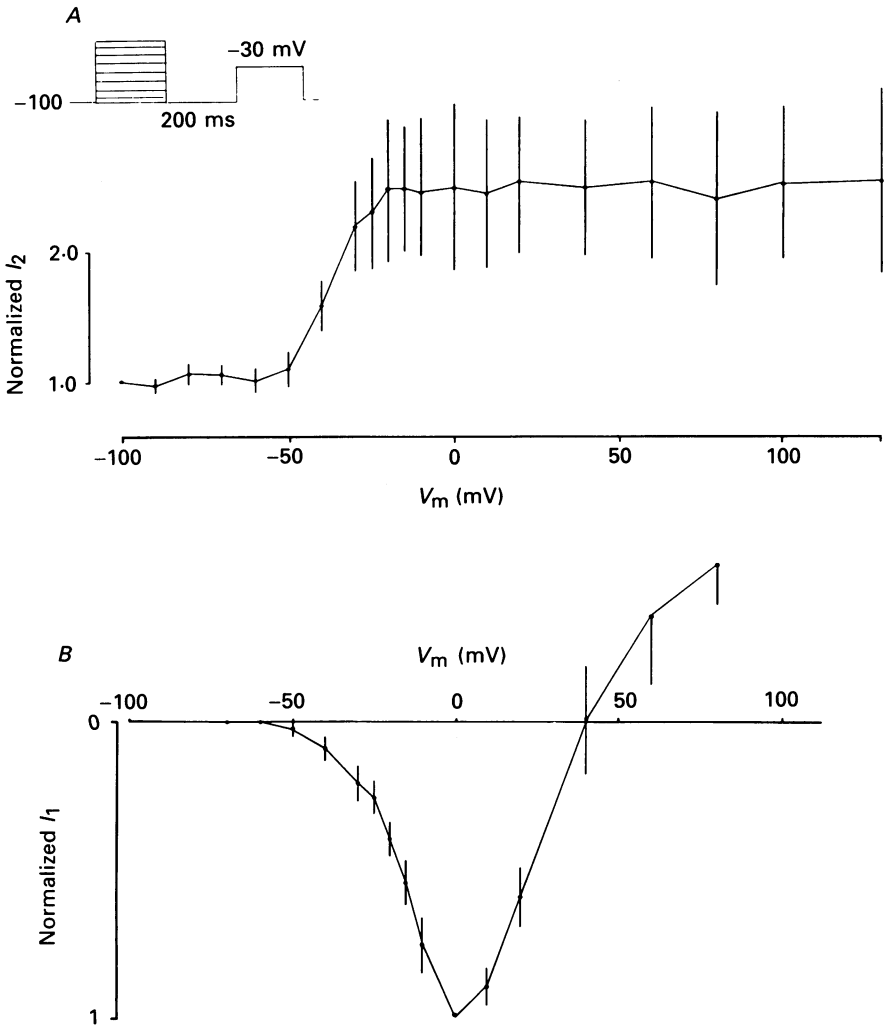

Fig. 5. Potentiation of peak Ba^{2+} current by 200 ms pre-pulses as a function of pre-pulse potential. The scheme of the voltage protocol is shown in the inset. Test pulse to −30 mV, pulse interval 200 ms. Mean ± S.D. from seven cells. *A*, the potentiation is expressed as the peak of the current with pre-pulse (I_2) divided by the current amplitude without a pre-pulse (pre-pulse potential −100 mV). *B*, *I–V* relationship, obtained from the same cells during the pre-pulses (I_1). Peak Ba^{2+} currents normalized by the current at 0 mV.

potentials, and close to the threshold inactivation was not complete during the 200 ms pre-pulse. To analyse the possible link between the potentiation of the T-type current by the pre-pulse and the transition of T-type channels to the inactivated

state, 10 s pre-pulses were used to cause steady-state inactivation. For vascular T-type channels to the inactivated state, 10 s pre-pulses were used to cause steady-state inactivation. For vascular T-type Ca^{2+} channels, the steady-state activation and inactivation curves do not overlap significantly (Akaike *et al.* 1989), so a correction for non-inactivated channels was not necessary.

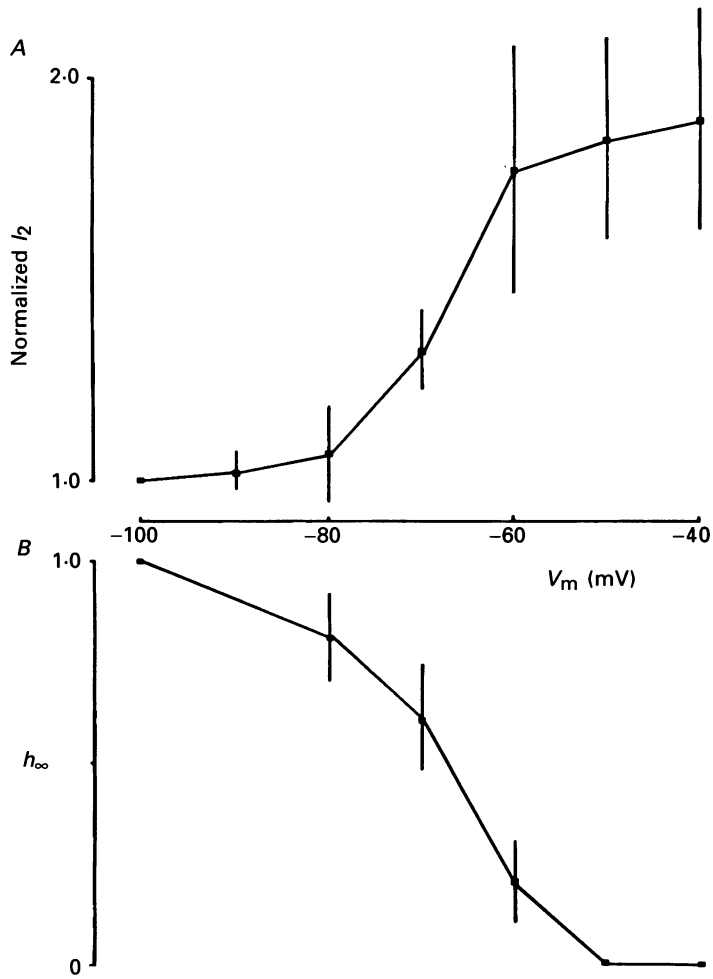

Fig. 6. Potentiation of the peak Ba^{2+} current by 10 s pre-pulses as a function of the pre-pulse potential. Ten second long pre-pulses were followed by a 300 ms repolarization to −100 mV and by 200 ms test pulses to −30 mV. Mean ± S.D. from seven cells. *A*, the potentiation is expressed as the peak of the current with pre-pulse (I_2) divided by the current peak without a pre-pulse (pre-pulse potential −100 mV). *B*, steady-state inactivation curve (h_∞); the peak Ba^{2+} current at −30 mV is plotted as a function of the holding potential.

Figure 6 shows the effect of 10 s pre-pulses to potentials between −90 and −40 mV on I_2. The curve is S-shaped; potentiation started at around −80 mV and showed saturation close to −50 mV. The results in Fig. 6*A* differ from those in

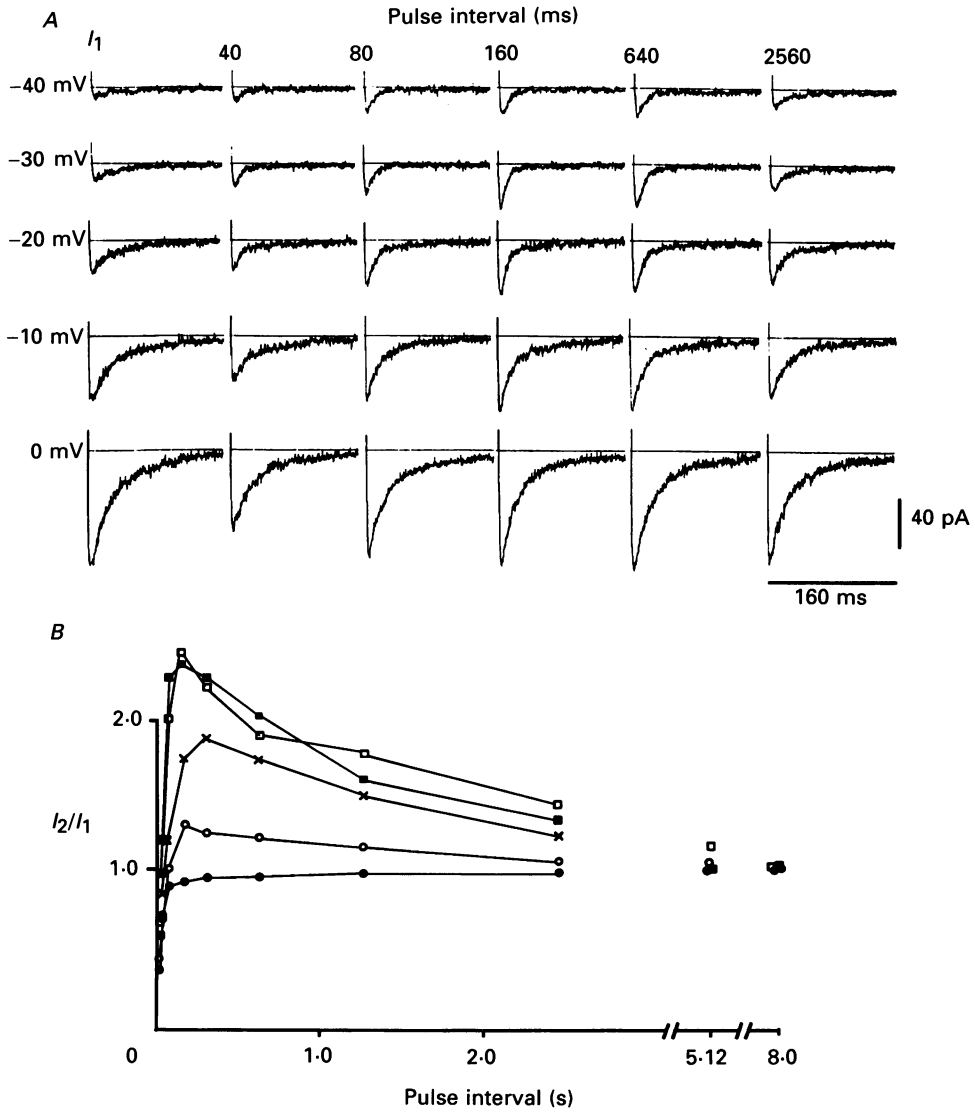

Fig. 7. Recovery of Ba^{2+} currents from short-term inactivation studied with a paired-pulse protocol. Both clamp steps to the same potential (indicated). Basal frequency of the pulse pair was 0·1 Hz. Holding and pulse interval potential −100 mV. The duration of the pulse interval varied between 20 ms and 8 s. *A*, current traces from computer play-backs. First trace of each row shows a representative Ba^{2+} current (I_1) in response to the pre-pulse, the potential of which is marked on the left. The following traces are the test currents (I_2), corresponding to the pulse intervals indicated at the top. *B*, for each pulse pair, the peak of the Ba^{2+} current during the test pulse (I_2) was divided by the peak of the Ba^{2+} current during the pre-pulse (I_1); the ratio I_2/I_1 is plotted as a function of the pulse interval. □, −40 mV pre-pulse; ■, −30 mV; ×, −20 mV; ○, −10 mV; ●, 0 mV.

Fig. 5*A* in that potentiation was already seen after pre-pulses to −80 or −70 mV, when the pre-pulses lasted 10 s instead of 0·2 s. The high correlation between the dependence of potentiation on the potential of the pre-pulse (Fig. 6*A*) and the voltage dependence of the steady-state inactivation (Fig. 6*B*) suggests, as a

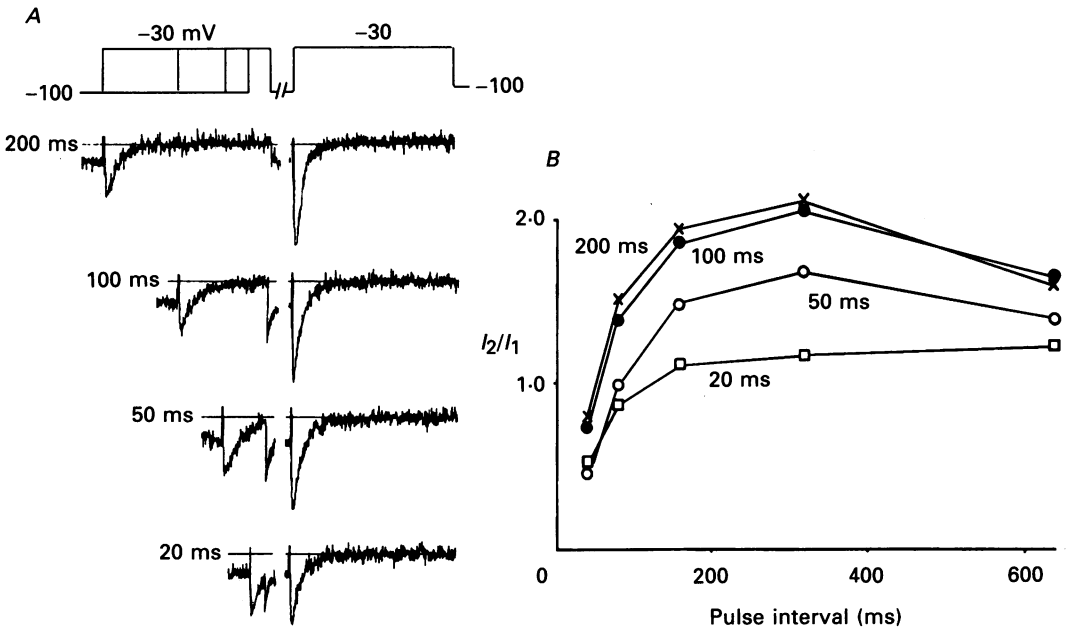

Fig. 8. Potentiation depends on the extent of inactivation during the pre-pulse. *A*, pre-pulse to −30 mV of variable duration was followed by a 160 ms interval to −100 mV and test pulse to −30 mV. The pre-pulse duration is indicated at the current traces. Note: the current trace during the pulse interval is interrupted. *B*, ratio I_2/I_1 plotted against pulse interval for different pre-pulse durations.

hypothesis, that potentiation and the transition to the inactivated state might be causally related.

Recovery of T-type Ba^{2+} currents from short-term inactivation

To study the time-dependent recovery of Ca^{2+} channels from inactivation, induced by a 200 ms pre-pulse, the interval between the pre-pulse and a test pulse of equal amplitude was varied between 20 and 8000 ms. When pulses to −40 or −30 mV from a holding potential of −100 mV were used, the current induced by the test pulse (I_2) reached the amplitude of the control current (I_1) within 20–80 ms. Intervals between 80 and 2560 ms increased I_2 beyond I_1 (Fig. 7*A*). After intervals as long as 8 s, I_2 and I_1 were almost identical, i.e. the potentiating effect of the pre-pulse disappeared. In Fig. 7*B*, the extent of potentiation is indicated by the peak ratio I_2/I_1 which is plotted against the duration of the pulse interval, for different pulse potentials. At pulse potentials of −40 or −30 mV, where the inward current was mainly T-type, a maximal potentiation of I_2 was observed with the pulse intervals of 160–320 ms; the effect decayed when the intervals were longer. When pulses to

−20 or −10 mV were applied, the potentiating effect of the pre-pulse was attenuated. With pulses to 0 mV, I_2 was always smaller than I_1, i.e. there was no potentiating effect of the pre-pulse, which is consistent with the fact that the T-type current forms only a small fraction of the total current at this potential and the main part of the current is carried through L-type calcium channels.

Since L-type Ca^{2+} channels are sensitive to Bay K 8644 and T-type channels are not, the recovery of I_{Ba} from short-term inactivation was studied in the absence and presence of this Ca^{2+} agonist. In the presence of Bay K 8644 (5 μM, 2 min) the current I_1 (at −20 mV) was increased by a factor of 3·5 and its time course of inactivation became slow (not illustrated). Under these conditions I_2 was always smaller than I_1, i.e. the potentiating effect was abolished. This can be explained by a strong enhancement of the L-type current at −20 mV in the presence of Bay K 8644 with the result that the time course of I_{Ba} recovery from inactivation was determined by the recovery of the L-type current.

The potentiation of T-type Ca^{2+} current by pre-pulses has not yet been reported in the literature; instead it has been reported that recovery from inactivation occurs without a transient facilitation. Most of these results were obtained at room temperature (Carbone & Lux, 1987*a*; Akaike *et al.* 1989; but see Hirano *et al.* 1989*a*; Tseng & Boyden, 1989). To test whether the potentiation occurs only at 36 °C, we studied and compared the recovery from inactivation at 24 and 36 °C. At −30 mV, a decrease of the temperature from 36 to 24 °C reduced the peak of the inward current to about half, slowed down the time course of inactivation and delayed the recovery. At 36 °C, the peak ratio I_2/I_1 reached its maximum of 2·1 after 160 ms intervals, whereas at 24 °C a maximal ratio of 1·8 was measured for intervals between 640 and 1280 ms (not illustrated). In conclusion, the potentiating effect of pre-pulses, although less obvious, was also observed at 24 °C.

The results of the paired-pulses experiments suggested a link between potentiation of T-type current and the transition of T-type calcium channels to the inactivated state (Fig. 6). In line with the above hypothesis one can expect that the pre-pulse potentiation becomes attenuated or abolished if the extent of inactivation is reduced, for example by reducing the duration of the −30 mV pre-pulses progressively. In Fig. 8, the pre-pulse duration was shortened from 200 to 100 ms, which had hardly any effect on the extent of potentiation. However, with the further shortening of the pre-pulse duration to 50 and 20 ms the potentiation became gradually reduced. Pre-pulses to −30 mV, longer than 200 ms, caused no further increase of potentiation, probably because at this potential inactivation reached completion within this period of time. This saturation does not conflict with the results showing stronger effectiveness of 10 s pre-pulses (Fig. 6) since inactivation was much slower when the pre-pulse potential was set to −70 or −80 mV. The results support the hypothesis that potentiation of T-type Ca^{2+} channels is causally related to the inactivated state.

Potential dependence of the recovery of T-type Ba^{2+} currents from inactivation

Since the inactivated channel is a closed channel, recovery from the inactivated to an available state is necessary before a T-type current and its potentiation can be observed. T-type Ca^{2+} channels recover from inactivation at a rate that increases at more negative potentials (Carbone & Lux, 1987*a*; Akaike *et al.* 1989; Hirano,

Fozzard & January, 1989*b*; Chen & Hess, 1990). Therefore, the dependence of potentiation on the potential during the pulse interval was studied, using the duration of the interval as parameter.

In an initial series of experiments, 200 ms pre-pulses were used for short-term inactivation. Figure 9 shows that the potentiation reached a maximum within

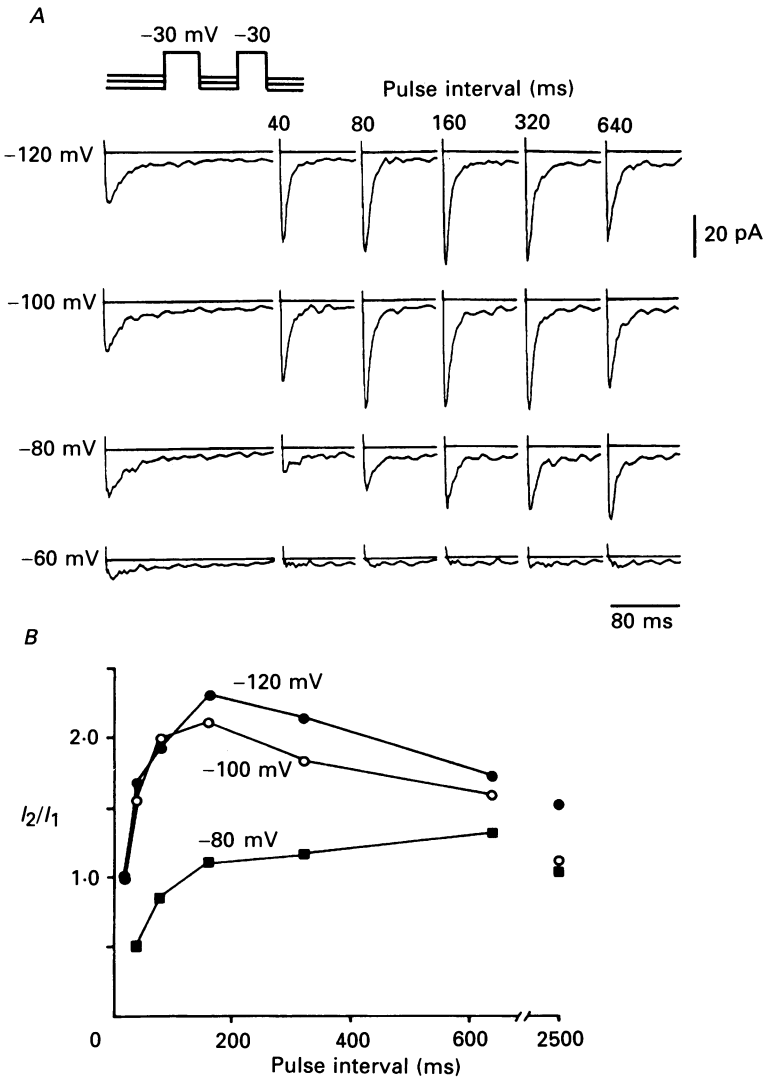

Fig. 9. Recovery of peak Ba^{2+} current from short-term inactivation at different holding potentials. Pre- and test pulses of 200 ms duration to −30 mV. *A*, original current traces; the holding potential during interval is indicated on the left. The first current of each row is a representative example of the current during the pre-pulse (I_1). *B*, I_2/I_1 (peak values) plotted against the duration of the interval for different holding potentials.

160 ms (with peak I_2 more than twice as large as I_1) for both potentials of −120 and −100 mV. At a potential of −80 mV, however, potentiation was found only after longer intervals (640–1280 ms) and the maximal degree of potentiation was smaller ($I_2/I_1 \approx 1{\cdot}3$). When the holding potential and the potential of the pulse interval were

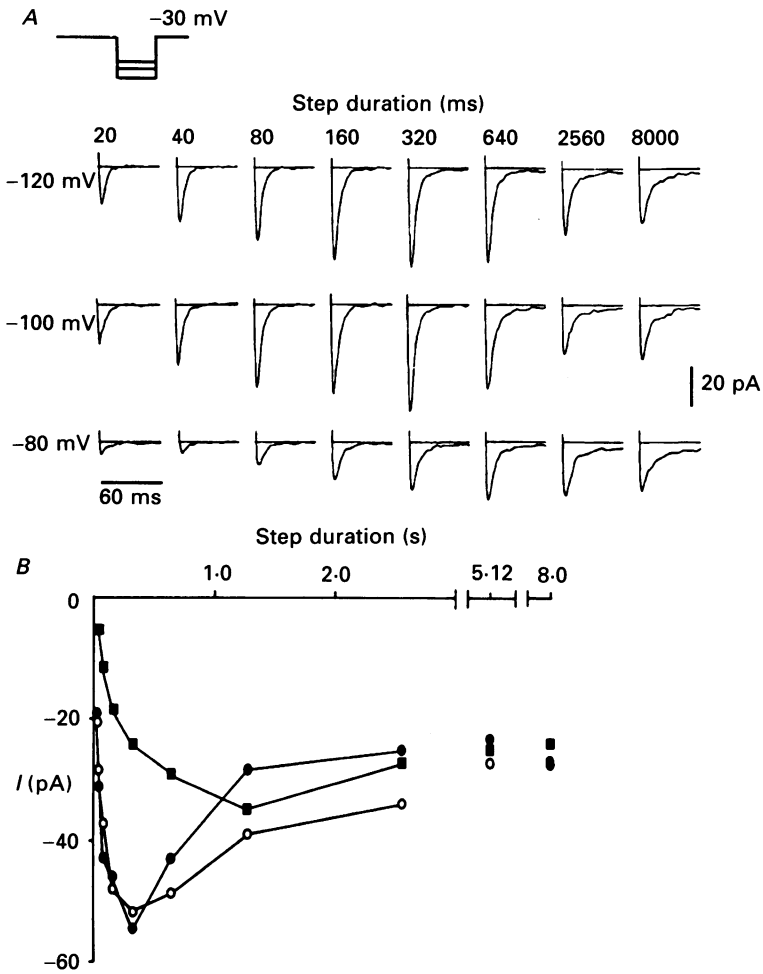

Fig. 10. Recovery of the peak Ba^{2+} current from long-term inactivation. Inset, pulse protocol. The membrane was held at −30 mV for 10 s. Voltage steps of different durations (indicated at the top) were applied to −120, −100 and −80 mV. Peak Ba^{2+} currents measured on returning to −30 mV. *A*, current traces; the potential of the step is indicated on the left. *B*, peak Ba^{2+} current is plotted against the duration of repolarization for different potentials of step: ■, −80 mV; ●, −100 mV; ○, −120 mV.

set to −60 mV, the channels remained largely inactivated and recovery of T-type currents could not be evaluated.

In a second series of experiments, the influence of the potential of the pulse interval on potentiation was studied after steady-state inactivation. In these experiments the membrane was held at −30 mV for 10 s, and the membrane

potential was stepped for different times to −80, −100 and −120 mV before returning to −30 mV, which was used as the test step inducing T-type currents (Fig. 10). Steps of 5·12 s were long enough to cause a steady-state recovery from inactivation, i.e. currents after 5·12 and 8 s steps had the same amplitude. The same peak current was also measured after a 40 ms step to −120 mV. When the hyperpolarization lasted 320 ms, the peak current exceeded the one after an 8 s step by a factor of 1·9 , both for steps to −120 and −100 mV. At −80 mV the maximal potentiation was only about 1·3 and required a step duration of 1·28 s. These results suggest that T-type Ca^{2+} channels recovered from short-term inactivation in a similar way as from long-term inactivation (Figs 9 and 10), in both cases with transient potentiation of the current.

Potentiated T-type current inactivates faster

Until now, potentiation was considered only with regard to the peak current. However, the current traces in Fig. 11*A* clearly show that the potentiated current I_2 inactivates faster than the control current I_1. For an analysis of the inactivation time course, minimal contamination with L-type Ca^{2+} currents was achieved by using intracellular solutions without Mg-ATP and without EGTA (see Methods section). The basic effect of pre-pulse potentiation was not modified by this solution. Figure 11*B* shows an exponential fit of the inactivation time course; the potentiated current I_2 inactivated with a time constant of 5·3 ms but the control current I_1 inactivated with a time constant of 13·6 ms. At a peak current ratio I_2/I_1 of $1{\cdot}98 \pm 0{\cdot}17$ the mean time constants obtained from six cells were $5{\cdot}6 \pm 0{\cdot}7$ ms for I_2 and $14{\cdot}5 \pm 1{\cdot}5$ ms for I_1. Faster inactivation resulted also in decrease of time-to-peak of the potentiated current (Fig. 11*B*).

Due to the faster inactivation time course, the potentiated current I_2 did not transport more charge than the control current I_1. Integration of the currents over time revealed that the ratio of the charge transported by I_2 (Q_2) over charge transported by I_1 (Q_1) was $0{\cdot}94 \pm 0{\cdot}16$. This may suggest that the T-type Ca^{2+} channels spend roughly the same time in the open state, independent of whether the current is potentiated or not. In Fig. 11*C*, the ratio of the charge (Q_2/Q_1) is plotted as a function of the duration of pulse interval. Unlike the current peak (I_2/I_1), the charge did not show a potentiation, i.e. it recovered without overshoot.

T-type Ca^{2+} channels recover faster from inactivation than L-type channels

Vascular smooth muscle cells from the coronary arteries have resting potentials ranging from −60 to −40 mV (Hirst & Edwards, 1989). At these potentials T-type Ca^{2+} channels are largely inactivated. However, the channels could become available during transient hyperpolarizations, e.g. due to activation of K^+ channels by release of intracellular Ca^{2+} or by neurotransmitters. Nevertheless, the physiological significance of T-type Ca^{2+} channels for Ca^{2+} influx is a matter of debate because recovery from inactivation was reported to be slower for T- than for L-type Ca^{2+} channels (Hirano *et al.* 1989*a*).

Recovery of T- and L-type channels from steady-state inactivation was compared in experiments where the membrane was held at potentials between −30 and 0 mV and repolarized to −100 mV for different durations (Fig. 12). Eight second intervals

were sufficient to reach a steady state of the availability of both T- and L-type current. For example, the tracings on the right of Fig. 12*A* show that the peak amplitude progressively increased when the potential was stepped to −30, −20, −10 and 0 mV. After a 320 ms interval, however, the peak current at −30 mV was

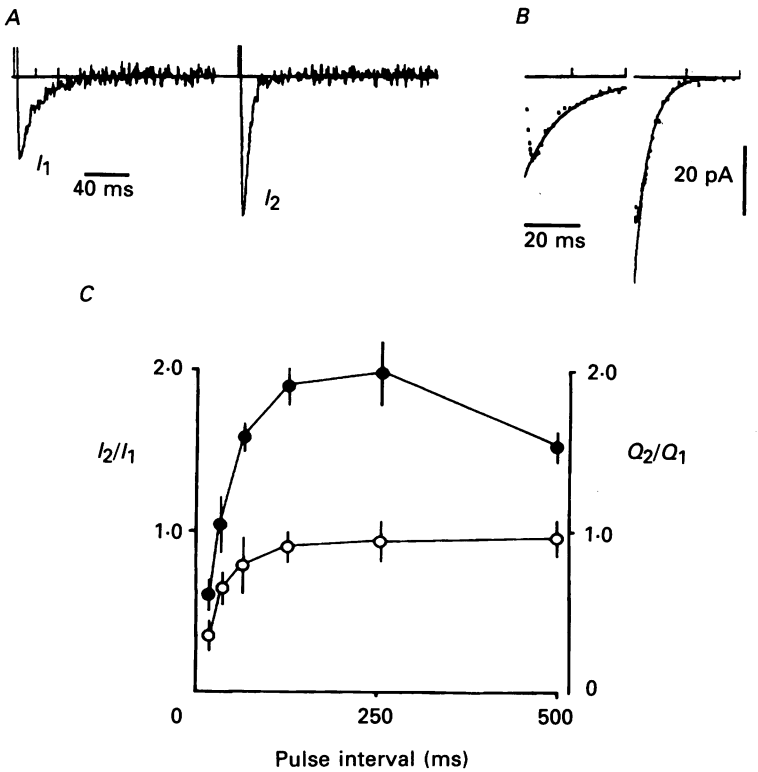

Fig. 11. Inactivation of potentiated T-type current. *A*, traces of Ba^{2+} currents during the pre-pulse (I_1) and the test pulse (I_2) to −30 mV. The duration of both pulses was 200 ms, the duration of the pulse interval 256 ms. *B*, currents from *A* fitted with single exponential functions; the time constants were 13·6 ms for I_1 and 5·3 ms for the potentiated current I_2. Note the expanded time scale. *C*, the effect of a pre-pulse on the peak current (ratio I_2/I_1, ●, left ordinate) and the time integral of the current (ratio Q_2/Q_1, ○, right ordinate) plotted against the duration of the pulse interval. Mean ± S.D. from six cells. To suppress L-type Ba^{2+} currents, intracellular solution was free of EGTA and ATP.

larger than that at 0 mV (Fig. 12*A* and *B*). At −20 mV, the current recorded after a 80 ms step to −100 mV had the same peak amplitude as after a 8 s step but inactivated faster. These results suggest that both T- and L-type Ca^{2+} channels contributed to the recorded current. At −30 mV, and after short hyperpolarizations, the contribution of L-type was low and the T-type current was predominant because it recovered faster than the L-type and with an overshoot (potentiation).

In conclusion, short hyperpolarizations were sufficient for recovery of the T-type Ca^{2+} channel from steady-state inactivation; the ability of calcium channels to change the membrane potential can be mainly attributed to T-type current after

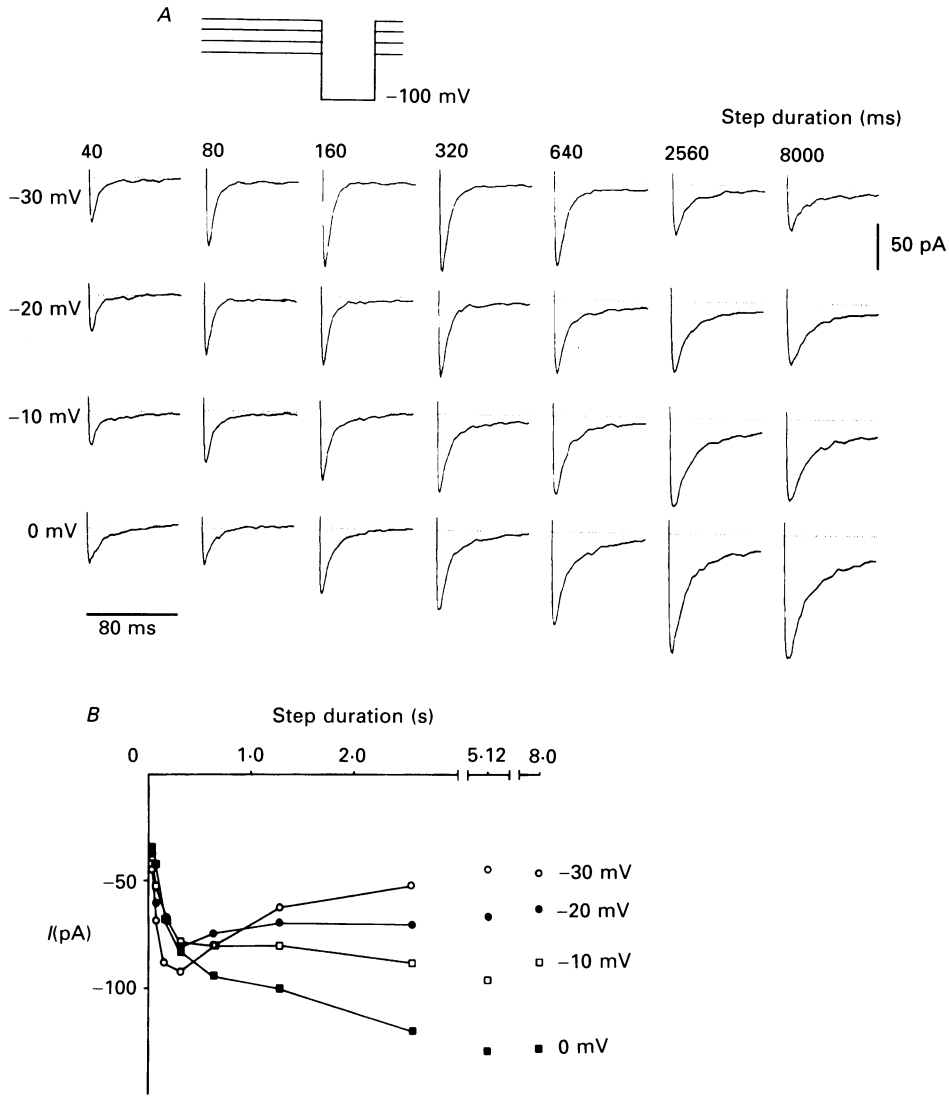

Fig. 12. The removal from steady-state inactivation is faster for T-type than for L-type Ca^{2+} channels. The inset shows the pulse protocol. The holding potential was varied between −30 and 0 mV. The potential was stepped to −100 mV for time intervals lasting between 20 ms and 8 s. The peak Ba^{2+} current was measured upon return to the holding potential. *A*, current traces. The holding potential is marked on the left. *B*, the peak Ba^{2+} current is plotted against the duration of the repolarizing step for different holding potentials.

short hyperpolarizations while longer hyperpolarizations were required for domination of L-type calcium current.

DISCUSSION

Potentiation is not due to superimposed outward currents

The results presented in this paper demonstrate that T-type Ca^{2+} currents can recover from inactivation exhibiting a transient state of potentiated peak current. Before models and implications are discussed, arguments shall be presented demonstrating that (1) the potentiation of peak inward current is not due to the depression of a time-dependent outward current and (2) it is the T- and not the L-type Ca^{2+} current that is potentiated.

The peak of inward current could be underestimated if it was contaminated by superimposed K^+ outward current with rapid activation–inactivation kinetics (for example the A-type K^+ current in smooth muscle cells from the portal vein: Beech & Bolton, 1989; ureter: Imaizumi, Muraki & Watanabe, 1990). If the increased pulse frequency decreased the peak outward current, the net inward current would be increased. In the present case, this possibility is most unlikely for the following reasons. The known vascular K^+ currents are small at negative potentials and they increase with more positive potentials. At positive potentials, however, no sign of outward currents with fast kinetics was recorded; most likely, the K^+ channels were effectively blocked by cell perfusion with Cs^+- or Na^+-rich solutions. Also, the potentiation of the peak inward current was restricted to potentials negative to -10 mV. Moreover, in experiments with K^+-rich intracellular solution no A-type K^+ current was found in these cells (authors' unpublished observations). To account for the faster inactivation of the potentiated peak current (Fig. 12), the hypothetical contaminating current should be initially outward and then inward. Putative time-dependent Cl^- currents are unlikely to contribute since the peak inward current and its potentiation were not affected by changes in $[Cl^-]_i$, expected to modify Cl^- currents. Removal of extracellular Ca^{2+} or Ba^{2+} ions abolished the peak inward current and its potentiation, as expected from these ions following through Ca^{2+} channels. Similarly, the blocking effect of $NiCl_2$ (0·5 mM, bath applied) is consistent with the idea that the peak inward current flows through Ca^{2+} channels.

The potentiation is due to T-type but not L-type Ca^{2+} channels

Although the recorded currents seem to be always a mixture of currents through T-type and L-type calcium channels, the useful proportion between them was found in the range of potentials between -40 and -20 mV, where the peak inward current as well as its potentiation was mainly attributed to a current through T-type Ca^{2+} channels. Between -40 and 0 mV, the T-type current has been reported to contribute a 'hump' to the *I–V* relationship (e.g. Akaike *et al.* 1989); in the present experiments, this 'hump' was indeed recorded, and it was augmented during potentiation by a pre-pulse (Fig. 3*B*). At positive potentials, where L-type channels are thought to be predominant, pre-pulses did not potentiate but rather depress the peak current. Also, the T-type current exhibits a steady-state inactivation that

starts at −80 mV and is complete at about −60 mV (Ganitkevich & Isenberg, 1990*a*, Fig. 10). Indeed, potentiation by 10 s pre-pulses became apparent at potentials positive to −80 mV and reached saturation at −50 mV, i.e. it followed the voltage dependence of the steady inactivation (Fig. 8). The hypothesis, that the peak inward current and its potentiation can be attributed to the T-type channels, is further supported by the following observations: L-type channels conduct Ba^{2+} better than Ca^{2+} ions whereas similar conductivities for Ba^{2+} and Ca^{2+} were reported for T-type channels (Nilius, Hess, Lansman & Tsien, 1985; Akaike *et al.* 1989). Here, the substitution of $BaCl_2$ by $CaCl_2$ in the bath changed neither the current peak nor its potentiation, suggesting the involvement of the T-type channels. Further, the inactivation is Ca^{2+} sensitive in the case of L- but not of T-type channels. In our experiments the inactivation time course was not modified by Ba^{2+} substitution for Ca^{2+}. Moreover, peak current and inactivation time course were insensitive to changes in $[Ca^{2+}]_i$ produced by removal or addition of EGTA and BAPTA to the intracellular solution, or to the bath application of caffeine.

Properties of the T-type current compared to those in other preparations

A potentiation of neuronal (as well as cardiac) T-type currents by increased stimulation frequency or by paired pulses has not been found. The reason may be that the neuronal and the vascular T-type channels are different proteins. Regarding other types of smooth muscle cells, we observed a potentiation of T-type Ca^{2+} currents very similar to the one described in this paper in myocytes from the urinary bladder of the guinea-pig, using either 10 mM-Ba^{2+} or 2·5 mM-Ca^{2+} as the charge carrier (unpublished). However, a recent study of T-type Ca^{2+} currents in cultured aortic smooth muscle cells described single exponential recovery from inactivation, within 250 ms after short-term inactivation and within 7 s after long-term inactivation (Akaike *et al.* 1989). No potentiation of T-type current was reported in this study. Partially, the difference may be attributed to experimental conditions used in this study (experiments were done at 22–25 °C). In our own experiments at 24 °C, maximal potentiation was reached after longer intervals and decayed slower than at 36 °C. Other differences in the experimental parameters may have contributed as well. Thus, we cannot offer a clear explanation at present.

Whereas evidence of potentiation of T-type Ca^{2+} channel current has not been published, potentiation of L-type currents, either by increased frequency or by pre-pulses, has been reported for several preparations. For example in cardiac ventricular myocytes, the potentiation of L-type current was current dependent (i.e. it depended on the amount of Ca^{2+} influx) and it could be blocked by intracellular EGTA; it has been attributed to $[Ca^{2+}]_i$-dependent phosphorylation of L-type Ca^{2+} channels (Argibay, Fischmeister & Hartzell, 1988; Tseng, 1988; Zygmunt & Maylie, 1990; but see Lee, 1987, 1989; Schouten & Morad, 1989, for alternative explanations). These results differ from those in the present study since interventions like removal of ATP, addition of caffeine, and elevation of $[Na^+]_i$ or $[H^+]_i$ changed neither the peak current nor its potentiation suggesting that involvement of an enzymatic step (like channel phosphorylation) is unlikely.

Possible mechanism of T-type channel potentiation

The following results obtained in this study indicated that potentiation crucially depends on voltage-dependent T-type channel inactivation: (1) the extent of potentiation and the steady-state inactivation curve followed a very similar voltage dependence (Fig. 8). (2) Diminution of inactivation by reducing the duration of the pre-pulses reduced in parallel the extent of potentiation. (3) The potentiated current with the larger peak amplitude inactivated faster than the non-potentiated current.

Single-channel recordings of T-type calcium channels from sensory neurons (Carbone & Lux, 1987*b*), ventricular myocytes (Droogmans & Nilius, 1989) and fibroblasts (Chen & Hess, 1990) suggested that macroscopic inactivation is the result of the late occurrence of bursts of T-type channel openings. In extrapolating to the present results, potentiation can be interpreted as a shorter mean first latency compared to that for channels in the non-potentiated state. The model predicts that in the potentiated state channel openings concentrate in a shorter period of time, i.e. the bursts of currents would sum and the peak current would increase. If macroscopic inactivation is due to microscopic activation, the potentiated current should inactivate faster. According to this model the channel recovers from inactivation by passing transiently through a closed state C* which is distinct from the closed state C determining the steady-state availability. We suggest that the channel can open from C* after a latency which is shorter than if it opens from C.

It would be most useful if the above hypothesis could be supported by measuring distribution of first latency openings experimentally. Despite large efforts, our experiments failed due to technical problems: (1) The amplitude of T-type current in a typical cell bathed in 10 mM-Ba^{2+} at 36 °C was 20–40 pA; in comparison with the single-channel current at 22 °C (Ganitkevich & Isenberg, 1990*a*) one estimates less than 100 T-type Ca^{2+} channels per cell. (2) To resolve the fast kinetics of the first latency, filtering has to be done at 2 kHz or higher. At 22 °C, the low signal-to-noise ratio often led to the detection of false events, hampering any type of analysis. At 36 °C, we failed to perform recordings sufficiently long to get the reliable data.

Is potentiation of T-type Ca^{2+} currents of physiological importance?

During the potentiation, the transported charge is not increased, hence Ca^{2+} influx and its consequences for the contractile state are not augmented. Rather, potentiated T-type channels have the capability to depolarize the membrane at a higher rate. For example, T-type Ca^{2+} channels were shown to contribute to the pacemaker depolarization in cardiac sino-atrial cells (Hagiwara *et al.* 1988). However, in the smooth muscle cells of large coronary arteries, action potentials do not occur and the resting potential is in the range between -60 to -40 mV where T-type Ca^{2+} channels are almost in the inactivated state. Thus, a contribution of T-type channels is unlikely if inactivation is not removed by hyperpolarizations. For example, acetylcholine (10 μM) was reported to hyperpolarize the circumflex coronary artery of the guinea-pig from -58 to -70 mV (Keef & Bowen, 1989), which can result in partial recovery of T-type channels from steady-state inactivation.

In contrast to larger arteries, the myocytes of small arteries and arterioles have more negative resting membrane potentials, at least *in vitro*, and can generate action

potentials (for review see Hirst & Edwards, 1989). Provided that Ca^{2+} channels of the T-type, with properties similar to those described in this paper, are present in the membrane of myocytes from arterioles, potentiation of T-type channels during a burst of action potentials can result in a faster rate of pacemaker depolarization and increased rate of spontaneous firing. Future direct recordings from these cells are needed to prove this suggestion.

We would like to thank Dr W. Melzer (Bochum) for his substantial criticism of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft.

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