Voltage-dependent potentiation of low-voltage-activated Ca^{2+} channel currents in cultured rat bone marrow cells

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1. The whole-cell patch-clamp technique was used to study Ca^{2+} channel currents in stromal cells of 7–10 day dexamethasone-treated and control rat bone marrow cultures. In saline containing either 108 mM Ba^{2+} or a 2·5 mM Ca^{2+}–1 mM Mg^{2+} mixture, most cells expressed both fast-inactivating, low-voltage-activated (LVA) and slow-inactivating, high-voltage-activated (HVA) currents.

2. Repeated application of 400 ms voltage steps to 60 mV above the holding potential (V_h, −90 mV in Ca^{2+}–Mg^{2+} mixture and −60 mV in Ba^{2+}) at a frequency $\geqslant$ 0·1 Hz resulted in a potentiation of the LVA component of the 2nd and subsequent currents.

3. LVA current potentiation was examined using a two-pulse (prepulse–test pulse) method. Prepulses to V_h + 150 mV induced an 80–100% increase in the amplitude of the LVA component of Ca^{2+} channel currents in saline containing either Ba^{2+} or Ca^{2+}–Mg^{2+}. This effect was also seen in non-dexamethasone-treated cultures.

4. Potentiation was not modified by omission of ATP and GTP from the pipette saline, and was not inhibited by extracellular application of the broad spectrum kinase inhibitors H-7 or RK252-a.

5. Potentiation was dependent on the amplitude and duration of the prepulse. Using the standard protocol, the threshold for potentiation was approximately V_h + 45 mV and saturation occurred at V_h + 150–180 mV. Further increases in prepulse amplitude did not modify potentiation. With a prepulse to +10 mV (Ba^{2+} saline) potentiation was half-maximal with a prepulse duration of 250–300 ms duration and saturated at 750–1000 ms.

6. Peak potentiation occurred 1–2 s after the prepulse. The time for total decay of potentiation varied from 10 to 90 s.

7. Voltage dependency of prepulse-induced potentiation did not resemble that of inactivation induced by similar prepulses.

8. Current kinetics, I–V relationship and sensitivity to blockade by Ni^{2+} and diphenylhydantoin of prepulse-recruited current resembled those of control LVA current.

9. The amplitude of prepulse-recruited current was positively correlated with control LVA current amplitude.

10. LVA currents supported regenerative potentials under current clamp. Repeated activation reduced spike latency.

11. It is suggested that current potentiation may be recruited physiologically, possibly in association with activation of stretch-sensitive channels, causing enhanced activation of HVA Ca^{2+} currents.

Osteoblast-like cells and cell lines from a number of sources have been shown to express voltage-operated Ca^{2+} channels (VOCCs), at least two types having been described. ROS 17/2.8 and UMR 106.01 osteosarcoma cells express high-voltage-activated (HVA) VOCCs (Duncan & Misler, 1989; Grygorczyk, Grygorczyk & Ferrier, 1989), whereas osteogenic MC3T3-E1 cells express a low-voltage-activated (LVA) VOCC with biophysical characteristics resembling the neuronal T-type (Amagai & Kasai, 1989). Acutely isolated primary calvarial cells express both a slowly inactivating HVA current that is enhanced by Bay K 8644 and a fast-inactivating LVA current that has electro-

physiological characteristics similar to the T-current (Chesnoy-Marchais & Fritsch, 1988)

The role of these channels in bone cells is yet to be elucidated. HVA VOCCs of ROS 17/2.8 cells may participate in the control of osteocalcin secretion (Guggino, Lajeunesse, Wagner & Snyder, 1989) and are modulated by vitamin D_3 (Caffrey & Farach-Carson, 1989; Yukihiro, Posner & Guggino, 1994). HVA and LVA channels of calvarial-derived cells are modulated by arachidonic acid, suggesting involvement of these channels in the effects of phospholipase A_2 activation by agonists (Chesnoy-Marchais & Fritsch, 1994). Another potential function of VOCCs in bone cells is in the transduction of mechanical stimuli, possibly in combination with mechanosensitive ion channels, which have been observed in various bone-derived cells and cell lines (Duncan & Misler, 1989; Davidson, Tatakis & Auerbach, 1990; Duncan, Hruska & Misler, 1992; Davidson, 1993).

Bone marrow includes a heterogeneous population of cells. Stromal cells of cultured bone marrow are an adherent, non-haemopoetic population which can be induced to differentiate into osteoblasts by a combination of exposure to the corticosteroid dexamethasone and manipulation of culture conditions to select adherent cell types (Maniatopoulos, Sodek & Melcher, 1988; Kasegai, Todescan, Nagato, Yao, Butler & Sodek, 1991). Such cultures provide a model for study of regulation of bone cell differentiation (Publicover, Thomas & El Haj, 1994). Marrow stromal cells cultured for 1–2 weeks express both a T-current-like LVA current similar to that of calvarial cells, and a HVA, dihydropyridine-sensitive VOCC (S. Publicover, M. Preston & A. El Haj, in preparation). In older marrow cells the HVA current is expressed alone but the LVA current is rapidly induced upon exposure to dexamethasone (Publicover *et al.* 1994).

During electrophysiological studies on bone marrow stromal cells we have regularly observed a potentiation of LVA current amplitude in response to repeated activation at low frequency (> 0·1 Hz; Publicover, El Haj & Thomas, 1993). Voltage-induced potentiation of L-type VOCCs, potentially leading to enhanced Ca^{2+} influx, has been reported in a number of cells. Processes underlying current potentiation include recruitment of new channels, alteration of channel characteristics and shift in voltage dependency of activation (Lee, 1987; Pietrobon & Hess, 1990; Artalejo, Dahmer, Perlman & Fox, 1991; Sculptoreanu, Scheuer & Catterall, 1993). However, voltage-dependent potentiation of LVA (T-type) VOCCs, which may enhance cell excitability and recruitment of other voltage-activated conductances, has been described only in arterial myocytes (Ganitkevich & Isenberg, 1991). In this communication we describe the characteristics of LVA current potentiation in stromal cells and discuss the possible significance of this effect.

METHODS

Tissue culture

Bone marrow cells from 120 g Wistar rats were prepared by the method of Maniatopoulos *et al.* (1988), as described previously (Publicover *et al.* 1994). Rats were first concussed. This was followed by cervical dislocation, an approved standard method of humane killing under the Animals (Scientific Procedures) Act of 1986. Cells were seeded at a density of approximately 3×10^4 cells ml^{-1} onto 35 mm plastic dishes (3 ml $dish^{-1}$) and maintained at 37 °C in a humidified atmosphere consisting of 95% air–5% CO_2. Culture medium was α-modified Eagle's medium with 15% (v/v) fetal bovine serum, ascorbate (50 μg ml^{-1}), B glycerophosphate (10 mM), penicillin (100 μg ml^{-1}), gentamicin (50 μg ml^{-1}) and fungizone (3 μg ml^{-1}). Dexamethasone when present was at a concentration of 10^{-8} M. Medium was changed after the first 24 h and after every 48 h thereafter, a procedure that removes the non-adherent, haemopoietic cells and thus enriches the stromal cell content of the culture. Culture medium was replaced by recording saline (without dexamethasone) 30–60 min before beginning recording.

Salines and chemicals

Recordings were carried out in either Ca^{2+}–Mg^{2+} saline (2·5 mM $CaCl_2$, 1 mM $MgCl_2$, 140 mM *N*-methyl-D-glucamine, 10 mM Hepes, pH adjusted to 7·6 with HCl) or Ba^{2+} saline (108 mM $BaCl_2$, 10 mM Hepes, pH adjusted to 7·6 with NaOH; maximum Na^+ content 3 mM; Chesnoy-Marchais & Fritsch, 1988). This recipe for Ba^{2+} saline was originally selected to allow direct comparison of our records with the LVA currents of rat calvarial cells (Chesnoy-Marchais & Fritsch, 1988). Current-clamp recordings were carried out in Na^+–Ca^{2+}–Mg^{2+} saline (140 mM NaCl, 2·5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, pH adjusted to 7·6 with NaOH). Tetrodotoxin (TTX; Sankyo, Japan) when present was 200–400 nM, but no evidence of TTX-sensitive currents was seen in any of the salines. Standard pipette saline contained 150 mM CsCl, 10 mM DL-glucose, 5 mM EGTA, 10 mM Hepes, 5 mM $MgCl_2$, 2 mM ATP (Sigma), 0·2 mM GTP (Sigma). pH was corrected to 7·3 with CsOH. For current-clamp recordings, pipette saline contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM Hepes, 2 mM ATP (K^+ backfill). H-7 (LC laboratories, Woburn, MA, USA) was made up immediately before use as a stock solution of 4 mM and diluted to the appropriate concentration. K-252a (Calbiochem) was made up as a stock solution in dimethyl sulphoxide and maintained at 4 °C. Diphenylhydantoin (DPH; Sigma) was made up as a concentrated stock (10 mM) in saline and diluted before use. All salts were Analytical Reagent or tissue culture grade.

Experimental protocols

Histochemical staining of both control and dexamethasone-treated dishes for alkaline phosphatase revealed that reaction product was mostly restricted to aggregated cells and those adjacent, the proportion of alkaline phosphatase-positive cells being higher in the dexamethasone-treated dishes (Publicover *et al.* 1994). Cells close to but not visibly attached to cell clumps were therefore selected for recording. Multinucleate and spindle-shaped cells were

avoided. LVA currents were expressed during a period between 5 and 18 days in culture (HVA currents were detectable from 5 days onwards). The proportion of cells expressing the LVA current and the mean amplitude of LVA currents were both increased in dexamethasone-treated cultures. Most recordings were therefore obtained from dexamethasone-treated, 7- to 10-day-old cell cultures. Similar results were obtained from cells in untreated cultures (see Results). All recordings were carried out at room temperature (20–21 °C) using the whole-cell variant of the patch-clamp technique. Currents were recorded using a Warner PC501a amplifier, with filter cut-off set at 2 kHz. Signals were passed to an IBM-compatible PC fitted with an S200 interface card and running Active (v2.0) voltage-clamp software (Intracel, Royston, UK), which was used for data recording and pulse generation. Filamented, borosilicate capillary (1·5 mm o.d.) was used for electrode manufacture (Clark GC 150TF; Clark Electromedical Instruments, Pangbourne, UK). Polished electrodes had resistances of 3–7 MΩ. Seals of 10–40 GΩ were achieved before breakthrough. Cell input resistances were usually of 1–5 GΩ (Publicover *et al.* 1994) and series resistance was usually 3–10 MΩ. Capacitance was compensated when possible, but sometimes exceeded the nominal 100 pF compensation range of the amplifier. This high capacitance may reflect the presence of gap junctions to other cells (Publicover *et al.* 1994). Records from cells which showed evidence of poor clamping (notched currents or unusually slow activation of LVA current) were assumed to reflect the presence of significant contacts with adjacent cells and were not used. Recordings were commenced within 1–3 min of breakthrough.

Cells were held (V_h) at −90 mV in Ca^{2+}–Mg^{2+} saline and at −60 mV in Ba^{2+} saline. The high divalent cation concentration in Ba^{2+} saline caused a shift in channel voltage sensitivity of approximately +30 mV (Chesnoy-Marchais & Fritsch, 1988; Publicover *et al.* 1994). Most records of current potentiation were obtained using a paired-pulse protocol consisting of a potentiating prepulse (400 ms) and a test pulse (100 ms). The interpulse interval was normally set to 4 s. Test pulses (except for *I–V* determinations) were to V_h + 60 mV, i.e. −30 mV (Ca^{2+}–Mg^{2+} saline) or 0 mV (Ba^{2+} saline). An interval of 30–60 s was allowed after each stimulation protocol to allow potentiation to subside before further recording. This interval was extended if test pulse amplitude failed to return to control levels within the time allowed. LVA current blockers (Ni^{2+} and DPH) were applied by rapid superfusion through a coarse micropipette (200–250 μm diameter) placed 50–150 μm away from the cell.

For investigation of LVA current inactivation, a 100 ms test pulse to V_h + 60 mV was again used but was preceded by inactivating pulses of either 400 or 1000 ms duration, with an interpulse interval of 10 ms.

Data analysis

Off-line analyses were performed using an IBM-compatible PC running Active (v2.0) software. All records were leak subtracted (except where otherwise stated), using hyperpolarizing pulses delivered at the beginning and end of stimulus routines.

At the test potentials used in this study HVA currents, when present, were close to activation threshold and usually showed negligible inactivation. LVA current amplitude could therefore be estimated from the difference between peak and end of pulse amplitudes. When HVA current inactivation was apparent LVA current amplitude was calculated from the difference between peak and peak + 60 ms values.

To reduce the effects of variation in control current amplitude on quantitative assessment of LVA current potentiation, enhanced currents were normalized to control current amplitude and expressed as a percentage by using the formula:

Potentiation =

((enhanced current − control current)/control current) × 100.

Potentiation values and time constants of current decay given in the text and shown in the figures are means ± S.E.M. Student's *t* test was used to compare potentiation under different conditions. Paired *t* tests were used to compare current amplitudes before and after potentiation, the effects of channel blockers on control and recruited current amplitudes and time constants of control, potentiated and recruited currents.

Log fits to decay of potentiation were carried out using the curve fitting routines in CA-Cricket Graph. Semilogarithmic plots were well fitted by a straight line for the period >30 s after induction of potentiation, but clearly diverged above this line during the first 10–20 s. Initial fits were therefore made to the later part of the decay (>30 s after induction) and the equation of the line was used to calculate deviation during the first 30 s. A second curve was then fitted to these values.

RESULTS

Approximately 90% (107/119) of dexamethasone-treated cells from which recordings were made expressed LVA VOCCs. In Ca^{2+}–Mg^{2+} saline, LVA VOCCs could often be recorded in isolation, or with only slight contamination from HVA VOCCs. Rapidly activating and inactivating Na^+ currents of the type seen in calvarial cells (Chesnoy-Marchais & Fritsch, 1988) were not seen in marrow stromal cells even in Na^+–Ca^{2+}–Mg^{2+} saline. LVA currents were not affected by TTX at 200–400 nM, a concentration which abolishes Na^+ currents in calvarial cells (Chesnoy-Marchais & Fritsch, 1988).

Current kinetics and voltage dependence of activation and inactivation of LVA currents resembled those of neuronal T-currents (Fig. 1*A* and *B*). Use of Ba^{2+} saline (108 mM $BaCl_2$, 10 mM Hepes) increased LVA current amplitude by approximately 70% (Fig. 1*C*), increasing mean maximum current from 95·6 ± 20·3 pA (n = 30) to 158·4 ± 28·6 pA (n = 28). Ba^{2+} saline also reduced HVA current inactivation, such that in cells expressing both currents the LVA current usually occurred on a sustained inward current. In this medium, the voltage dependence of both activation and steady-state inactivation was shifted by approximately +30 mV (Chesnoy-Marchais & Fritsch, 1988; Publicover *et al.* 1994).

Repeated activation

Repeated application of 400 ms depolarizing pulses (0 or +10 mV at a frequency of 0·2 Hz) to dexamethasone-

treated marrow cells bathed in Ba^{2+} saline ($V_h = -60$ mV) induced clear potentiation (>20%) of LVA currents evoked by the second and subsequent pulses in 76% (19/25) of cells expressing the current (Fig. 2). For steps to +10 mV the second current was enhanced by $30 \pm 4\%$ ($P < 0{\cdot}001$, paired t test, $n = 25$) and the third current by $37 \pm 5\%$ ($P < 0{\cdot}001$, paired t test, $n = 19$). Subsequent currents in series were further potentiated, the effect saturating after five to ten activations. Repeated application of shorter pulses, to the same voltage, was markedly less effective (Fig. 2*B*). Steps to negative potentials did not induce potentiation. Repeated activation of the HVA current failed to cause any enhancement of amplitude, even upon steps to +70 mV.

Potentiation by prepulses

To allow characterization of the potentiating effect of repeated activation, a two-pulse (prepulse–test pulse) protocol was used. Prepulses (400 ms) were followed after 4 s by a 100 ms test pulse to $V_h + 60$ mV. In Ca^{2+}–Mg^{2+} saline ($V_h = -90$ mV) mean control LVA current amplitude in response to test pulses (to −30 mV) was $80{\cdot}7 \pm 17{\cdot}2$ pA ($n = 30$). Application of a prepulse induced an increase in LVA current amplitude in all cells expressing the current, but HVA currents were not affected (Fig. 3*A*). Mean potentiation in response to a prepulse to +60 mV was $76 \pm 16\%$ (range, 20–140%) of control amplitude ($n = 8$; $P < 0{\cdot}005$, paired t test).

Requirement for high energy phosphates and kinase activity

Omission of GTP and ATP from the pipette saline did not significantly modify the amplitude of potentiation. Potentiation induced by a prepulse to +60 mV (Ca^{2+}–Mg^{2+} saline) was $103 \pm 24\%$ when using pipette saline without GTP and $98 \pm 25\%$ when neither GTP nor ATP were

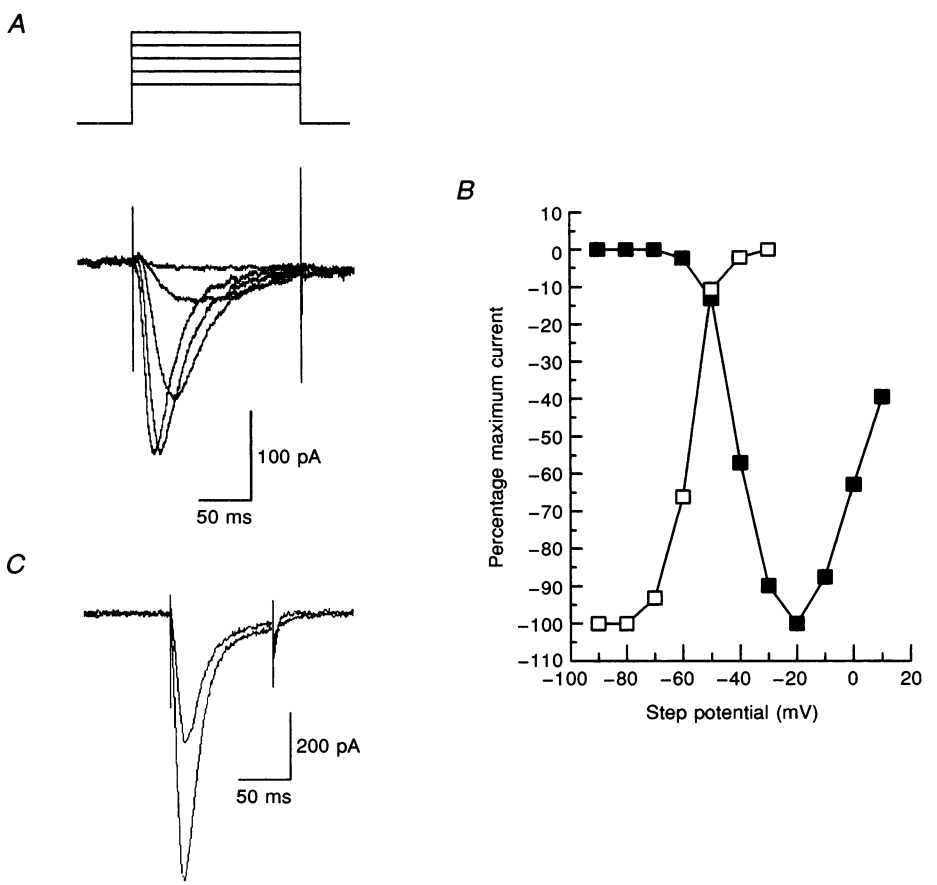

Figure 1. Characteristics of the LVA current

A, family of currents from a cell bathed in Ca^{2+}–Mg^{2+} saline. Cell was held at −90 mV and stepped to −60, −50, −40, −30 and −20 mV. *B*, voltage dependencies of peak LVA current amplitude (■) and reduction of LVA current amplitude by inactivation (□). Currents for investigation of inactivation were induced by stepping to −30 mV and preceded by inactivating steps of 1 s duration with a 10 ms interpulse interval. Recording conditions were as in *A*. *C*, enhancement of LVA current upon replacement of extracellular Ca^{2+}–Mg^{2+} saline (2·5 mM Ca^{2+}, 1 mM Mg^{2+}) with Ba^{2+} saline (108 mM Ba^{2+}). V_h and step potentials were −90 and −30 mV in Ca^{2+}–Mg^{2+} saline and −60 and 0 mV in Ba^{2+} saline.

included in the saline ($P < 0{\cdot}001$, $n = 14$ and $P < 0{\cdot}05$, $n = 8$, respectively). Potentiation persisted in cells dialysed with such ATP/GTP-free pipette backfill, even when held in whole-cell clamp for up to 45 min. Series resistance was seldom above 5 MΩ, a value that has been shown to be correlated with effective dialysis of whole-cell-clamped calvarial osteoblast-like cells (Chesnoy-Marchais & Fritsch, 1989).

Bath application of the broad spectrum kinase inhibitor H-7 also failed to inhibit potentiation. Potentiation of the LVA current in the presence of 250 μM H-7, determined 4 s after a 400 ms prepulse to +60 mV (Ca^{2+}–Mg^{2+} saline) was 86 ± 13% ($P < 0{\cdot}02$, paired t test, $n = 9$, not significant (n.s.) compared with non-H-7-treated cells; Fig. 3*B*). Comparison of potentiation induced by prepulses to +60 mV applied to cells both before and after exposure to H-7 (400 μM) revealed no modification of current recruitment ($P > 0{\cdot}8$, paired t test, $n = 7$). Application of 1 μM K-252a was similarly ineffective ($P > 0{\cdot}8$, paired t test, $n = 6$).

Use of Ba^{2+} as charge carrier

Use of Ba^{2+} saline did not modify the efficacy of prepulses in recruiting LVA current. (Fig. 3*C*). After compensation for the 30 mV shift in voltage sensitivity of the channels ($V_h = -60$ mV, test pulse to 0 mV; see Methods) the mean potentiation in response to a prepulse to +90 mV was 85 ± 12% ($P < 0{\cdot}0005$, paired t test, $n = 28$).

Since potentiation was fully expressed using Ba^{2+} as charge carrier and was not modified by the use of ATP/GTP-free pipette saline, all subsequent recordings were carried out under these conditions unless stated otherwise. Thirty-six cells which were exposed to similar prepulses (+90 mV for 400 ms, 4 s interpulse delay) at various times during these subsequent investigations showed similar levels of potentiation (92 ± 20%; range, 27–400%).

Prepulse parameters

To investigate the significance of prepulse parameters in determining the level of potentiation, the amplitude and duration of the prepulse were systematically varied whilst

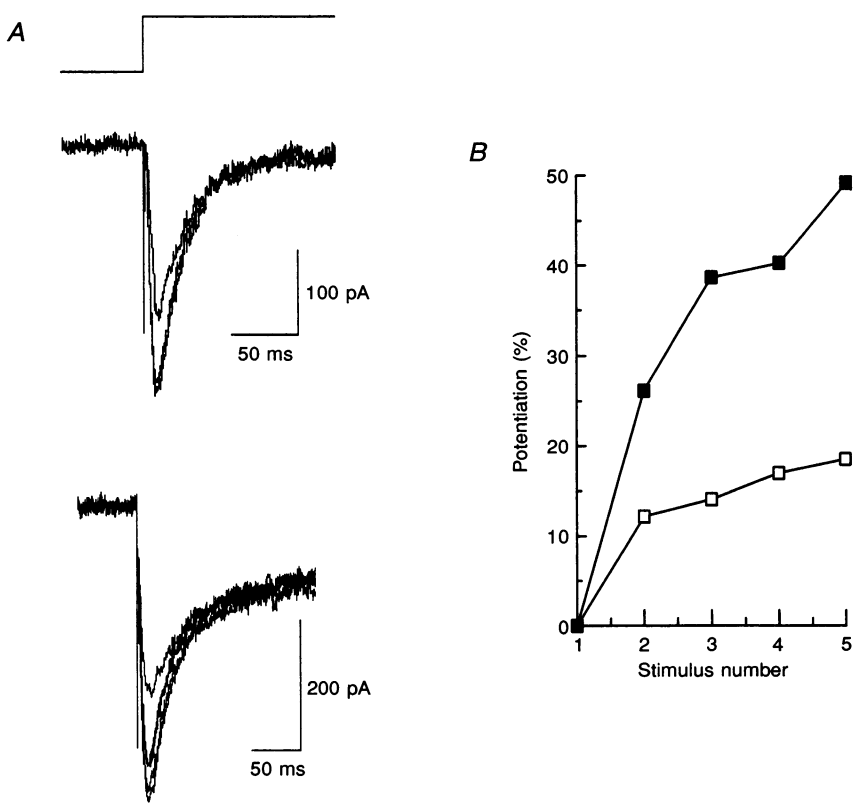

Figure 2. Potentiation of LVA current upon repeated activation

A, currents elicited in cells bathed in Ba^{2+} saline by repeatedly stepping from −60 to +10 mV at a frequency of 0·2 Hz. Upper panel shows three consecutive currents from a cell expressing only the LVA current. Lower panel shows five consecutive currents from a cell expressing both LVA and HVA currents. The second and subsequent LVA currents are potentiated but the non-inactivating component does not potentiate. *B*, effect of voltage step duration on response to repeated activation. Potentiation of LVA currents in response to repeated application of voltage pulses of 100 ms (□) or 400 ms (■) duration to the same cell. Voltage steps were applied at 0·2 Hz. Recording conditions were as in *A*.

maintaining the test pulse and interpulse interval at their standard values (see Methods). The magnitude of LVA current potentiation was clearly dependent on prepulse potential. Twenty-eight cells bathed in Ba^{2+} saline were each exposed to a series of prepulses ranging from −30 mV to 90 or 120 mV in 30 mV increments. The threshold for potentiation was between −30 and 0 mV and the effect increased sigmoidally with more positive prepulses (Fig. 4*A*). Saturation occurred at +90 to +120 mV and further increases in prepulse potential (in some cells up to +180 mV) did not modify the effect (Fig. 4*B*). In Ca^{2+}–Mg^{2+} saline, the voltage dependence of potentiation was shifted by −20 to −30 mV, threshold being between −60 and −30 mV (Fig. 4*A*) and saturation occurring at +60 to +90 mV.

Eight cells bathed in Ba^{2+} saline were tested using prepulses of constant amplitude (to +10 mV) but with prepulse duration varying between 0 and 1000 ms. All cells showed a clear sensitivity to prepulse duration. Potentiation was half-maximal at 200–300 ms and saturated at 750–1000 ms (Fig. 4*C*). Further increasing the prepulse duration, up to 1·5 s, did not modify the amplitude of potentiation.

Time course of potentiation

To investigate the time course of potentiation and the possible interaction of potentiation and LVA current inactivation, five cells were tested using a 400 ms prepulse to 0 mV (Ba^{2+} saline) and a range of prepulse–test pulse intervals (0·5, 1, 2, 3 and 4 s). An effect of the prepulse–test pulse interval was clearly discernible. When cells were held at the normal V_h for Ba^{2+} saline of −60 mV, maximal potentiation occurred after a 1 s delay in three out of five cells examined and at 2 and 3 s, respectively, in the other two. Mean potentiation was maximal at 1 s (Fig. 5*A*). Reduction of the delay to 500 ms greatly reduced potentiation ($P < 0·05$, paired t test) and with interpulse delays longer than 1 s the mean potentiation slowly decayed (Fig. 5*A*). When the same cells were held at −50 mV potentiation at 1 s was reduced (n.s., paired t test) and the maximum response occurred at 2–3 s in all the cells (Fig. 5*A*).

For investigation of the time course of extinction of the effect, ten cells were held at −60 mV (Ba^{2+} saline) and 100 ms test pulses to 0 mV were applied continuously at 0·1 Hz, a procedure that induced a stable but very low level

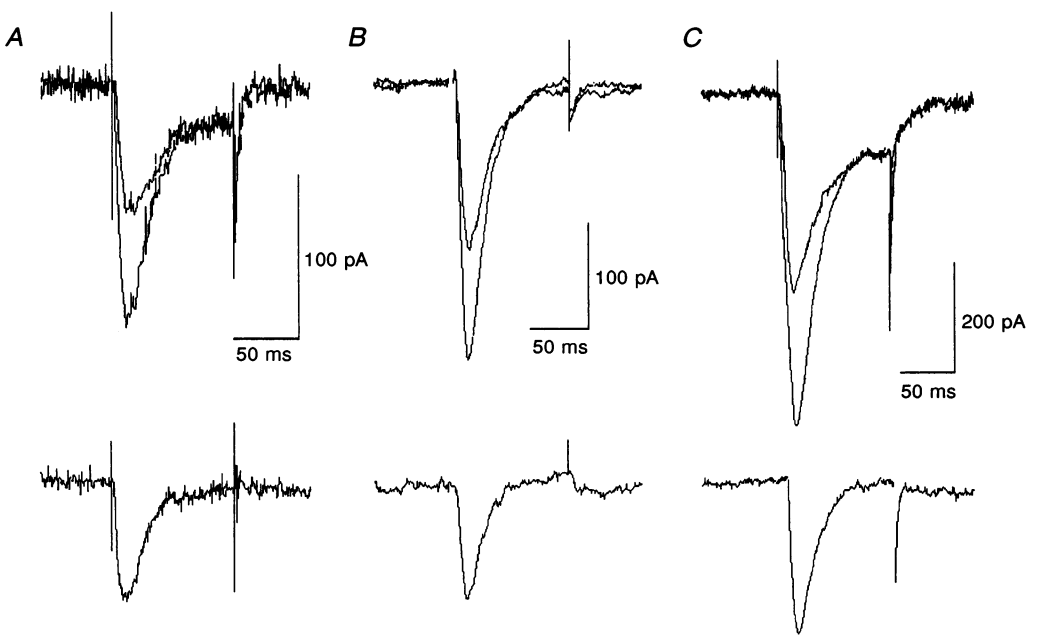

Figure 3. Induction of LVA current potentiation by prepulses

A, upper panel shows two superimposed currents induced by a 100 ms step from −90 to −30 mV (Ca^{2+}–Mg^{2+} saline) under control conditions and when preceded by a 400 ms prepulse to 60 mV. Interpulse interval was 4 s. Lower panel shows recruited current, obtained by subtraction of control from potentiated current. *B*, similar protocol to *A* but carried out in the presence of 400 μM H-7. *C*, similar protocol to *A* but cell was bathed in Ba^{2+} saline, for which V_h and voltage pulse potentials were adjusted by +30 mV (see Methods). The currents in this cell include a non-inactivating (HVA) component which did not potentiate.

(<10%) of potentiation. Strong potentiation was induced by a single large prepulse (1000 ms step to +60 mV) and the decay back to baseline was monitored. Potentiation was induced 2 or 3 times in each cell. The time course of decay was consistent between repeats (Fig. 5*B*) so a mean decay curve was calculated for each cell. In eight of these cells potentiation lasted 30 s or more (Fig. 5*B*). Semilogarithmic plotting indicated the presence of two components of decay. Six of the cells had two clearly separable components (Fig. 5*C*) with time constants of 9·4 ± 0·5 and 43·5 ± 5·0 s. These two components contributed nearly equally to potentiation determined 4 s after the prepulse. In each of the other four cells only one component was discernible. Two cells appeared to possess only the fast component of decay (time constants, 10·1 and 13·3 s), and in two cells only the slow component was present (time constants, 35·7 and 37·2 s).

Voltage dependencies of potentiation and inactivation

Prepulse-induced facilitation of T-currents in coronary artery myocytes has a voltage dependency which closely parallels that of inactivation (Ganitkevich & Isenberg, 1991). However, in the current study, voltage dependencies of potentiation and inactivation appear to differ (Figs 1 and 4*A*). To directly compare the voltage dependencies of these two processes, the LVA currents of three cells bathed in Ba^{2+} saline were recorded during the induction of both potentiation and inactivation. A test pulse was preceded by 400 ms prepulses with either a 10 ms (inactivation) or 4 s (potentiation) interpulse interval. In all three cells the voltage dependencies of the two processes clearly differed (Fig. 6). Prepulse potentials for half-maximal inactivation and current recruitment were −31·3 ± 2·0 mV and 10·5 ± 4·1 mV, respectively ($P < 0·02$, paired t test, $n = 3$).

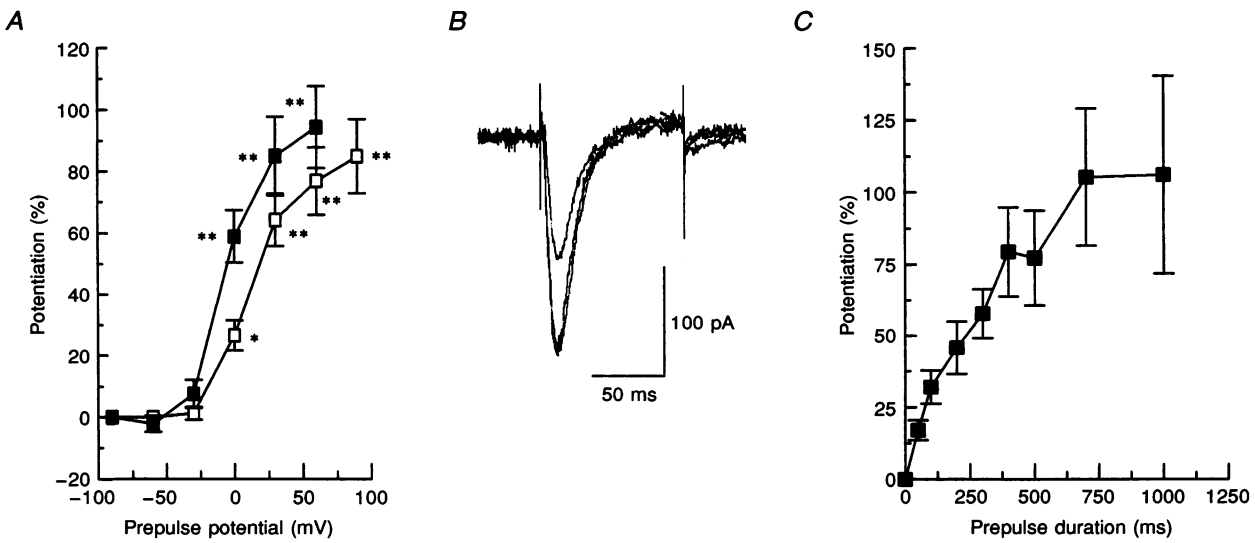

Figure 4. Effect of prepulse parameters on potentiation

A, effect of prepulse potential on potentiation. ■, mean response (bars, ± S.E.M.) of 30 cells bathed in Ca^{2+}–Mg^{2+} saline. V_h was −90 mV, test pulses (100 ms) were to −30 mV and prepulses (400 ms) were to −90 (zero prepulse), −60, −30, 0, 30 and 60 mV. □, mean response (bars, ± S.E.M.) of 28 cells bathed in Ba^{2+} saline for which V_h was −60 mV, test pulses (100 ms) were to 0 mV and prepulses (400 ms) were to −60 (zero prepulse), −30, 0, 30, 60 and 90 mV. Interpulse delay was 4 s. Asterisks show significance levels of paired t tests comparing potentiated with control (zero prepulse) currents. * $P < 0·001$, ** $P < 0·0005$. Unmarked points were not significant ($P > 0·05$). *B*, saturation of the voltage dependency of potentiation. Three superimposed traces show control current, current potentiated by a 400 ms prepulse to +90 mV and current potentiated by a 400 ms prepulse to +180 mV. Interpulse delay was 4 s. Ba^{2+} saline, $V_h = -60$ mV, test pulses to 0 mV. Most of the cells in which potentiation was investigated expressed only the LVA VOCC or both LVA and HVA VOCCs. A sustained outward current, which has not yet been characterized, is clearly discernible in these traces and occurs in approximately 66% of cells (Publicover *et al.* 1994). Neither the presence nor the amplitude of potentiation was dependent on expression of the HVA VOCC or the outward current. *C*, effect of prepulse duration on potentiation. Prepulses (to +10 mV) of 0–1000 ms duration preceded the test pulse (100 ms, 0 mV) by 4 s. Mean ± S.E.M. of 8 cells. Ba^{2+} saline, $V_h = -60$ mV.

Characteristics of recruited current

Prepulse-recruited currents were isolated by subtracting potentiated from control currents. Inactivation of these currents was slightly faster than that of controls. Current decay was fitted with a single exponential, the time constants being 16·8 ± 0·7 ms for control currents, 15·9 ± 0·3 ms for potentiated currents ($n = 8$; n.s. compared with control currents, paired t test) and 13·4 ± 0·8 ms for recruited currents ($n = 8$; $P < 0·05$ compared with control, paired t test). Current–voltage relationships of LVA currents were obtained from four cells (Ba^{2+} saline, $V_h = -60$ mV) by using 100 ms test pulses to a range of voltages, both under control conditions and preceded by a 400 ms prepulse to +30 mV. Figure 7*A* shows mean I–V curves for control and recruited currents, normalized to the current evoked at 0 mV (threshold for the HVA current). Activation thresholds and current amplitudes at negative test pulse voltages were similar, but in response to test pulses to voltages greater than 0 mV the recruited current was significantly smaller.

A number of cells were exposed to Ni^{2+} and to DPH, semi-specific blockers of LVA currents, to examine the relative sensitivity of control and recruited currents to these treatments. Potentiation was evoked by a step to +100 mV (Ba^{2+} saline) both under control conditions and in the presence of blockers. Exposure to $NiCl_2$ (33 and 60 μM) caused inhibition of the control LVA current (34 ± 7%, $n = 9$ and 68 ± 13%, $n = 4$, respectively) but did not inhibit HVA currents. Recruited currents were inhibited to a similar degree at both concentrations (33 ± 11%, $P = 0·94$, $n = 9$ and 55 ± 17%, $P = 0·65$, $n = 4$; paired t tests; Fig. 7*B*). Control and recruited LVA currents were also similarly inhibited by DPH. At 30 μM, DPH inhibited control and recruited currents by 29 ± 4 and 33 ± 8%, respectively ($n = 7$, $P = 0·60$, paired t test) and 100 μM DPH inhibited control and recruited currents by 53 ± 17% and 52 ± 13%, respectively ($n = 3$, $P = 0·96$, paired t test). Both currents were resistant to inhibition by nifedipine (4 μM, data not shown).

Relationship between control and recruited current amplitudes

During the course of this investigation sixty-four cells, bathed in Ba^{2+} saline, were exposed to a +90 mV prepulse applied as part of a standard prepulse protocol. To determine whether there was any relationship between control and recruited current amplitudes, the data from

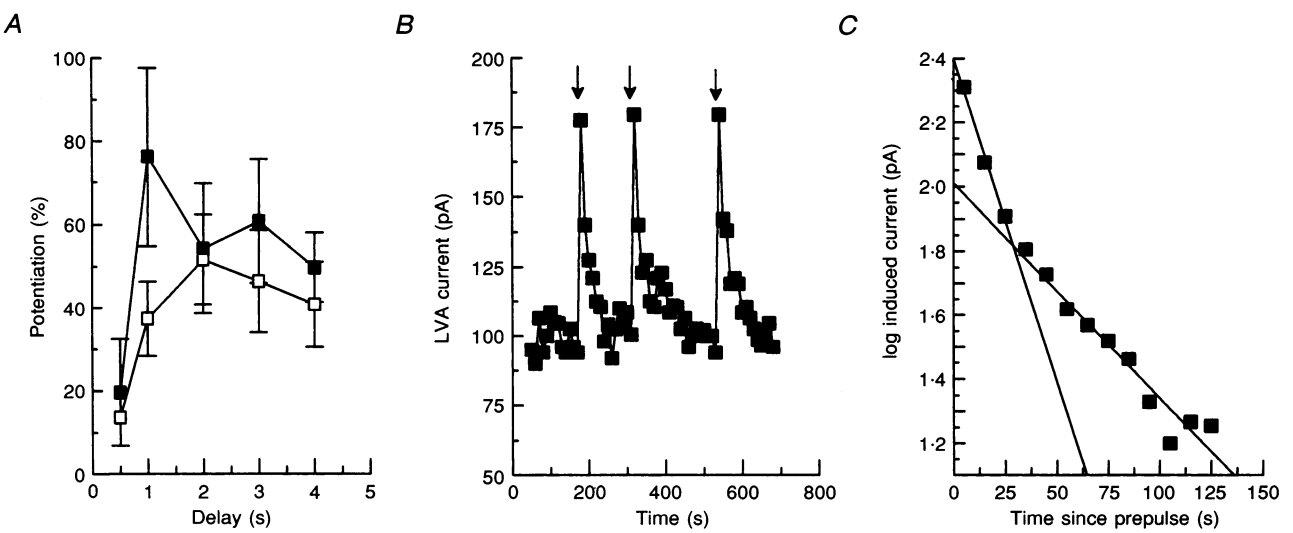

Figure 5. Time course of potentiation

A, effect of interpulse delay on amplitude of potentiation. Five cells were given 400 ms prepulses to +40 mV, followed by 100 ms test pulses to 0 mV, with interpulse intervals of 0·5, 1, 2, 3, and 4 s. ■, mean potentiation (bars, ± S.E.M.) with a V_h of −60 mV. □, mean potentiation (bars, ± S.E.M.) for the same five cells held at −50 mV. Ba^{2+} saline. *B*, time course of extinction of potentiation. LVA current amplitude was monitored continuously by application of test pulses (0 mV, 100 ms) at 0·1 Hz. Potentiation was induced 3 times. On each occasion a 1 s step to +60 mV (arrows) was applied midway between two test pulses. Ba^{2+} saline. *C*, semilogarithmic plot of decay of potentiation. Potentiation was induced 3 times (protocol as in *B*) and recruited current amplitudes were calculated by subtraction. The three sets of data for decay of recruited current were averaged and plotted semilogarithmically. Regressions to the two components were fitted using CA-Cricket Graph.

these cells were pooled and plotted as a scattergram. The fitted regression of recruited current on control current showed a positive correlation between data pairs ($y = 0{\cdot}25x + 41{\cdot}0$; $r = 0{\cdot}44$, $P < 0{\cdot}001$).

Current-clamp records

Mean membrane potential of cells held under current clamp in Na^+–Ca^{2+}–Mg^{2+} saline was $-46{\cdot}4 \pm 3{\cdot}5$ mV ($n = 34$). However, potentials were distributed bimodally with peaks at −30 and −55 mV. Fifty-nine per cent of cells (20/34) had potentials in the range −50 to −80 mV. LVA currents in these cells were only partially (up to 50%), if at all, inactivated. From a resting potential of −80 mV, maintained by current injection when necessary, half of the cells tested (16/30) displayed regenerative activity in response to depolarizing current injection (Fig. 8*A*). These spikes were seen in cells showing no Na^+ current and were not blocked by TTX (400 nM). Spike threshold appeared to be between −40 and −45 mV and the peak was between 0 and +10 mV. Spikes often had flattened tops, suggesting activation of outward current at around 0 mV (see Discussion). Spikes could also be induced upon relaxation of membrane potential after injection of hyperpolarizing current in cells with small membrane potentials (Fig. 8*B*). Repeated injection of depolarizing current, applied at 0·33 Hz, caused a slight enlargement of the spike and a marked reduction in latency in some cells (Fig. 8*C*).

Dexamethasone and potentiation

The data described above were obtained from cells previously treated with dexamethasone. To investigate any role of dexamethasone treatment in the potentiating effect, cells from non-dexamethasone-treated cultures were tested for prepulse-induced potentiation (Ba^{2+} saline, 400 ms

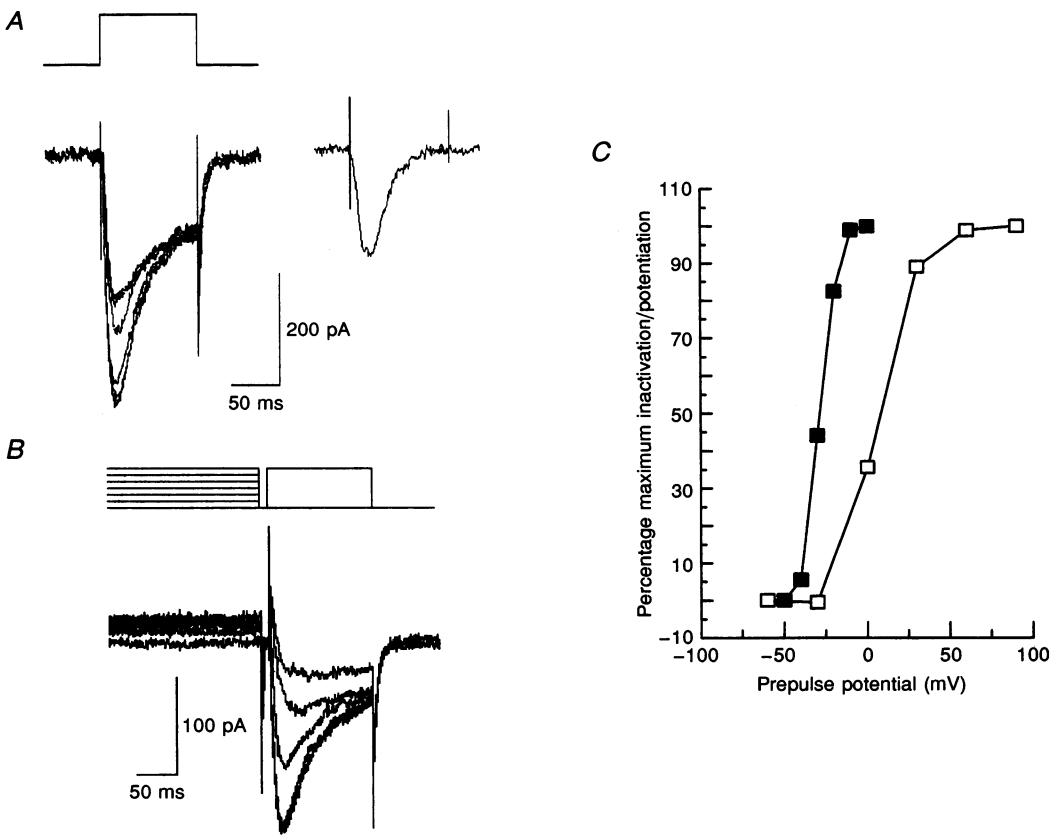

Figure 6. Voltage dependencies of potentiation and inactivation

A, six superimposed traces showing potentiation of currents induced by test pulses to 0 mV (Ba^{2+} saline, $V_h = -60$ mV) preceded by prepulses (400 ms) to −60 (zero prepulse), −30, 0, 30, 60 and 90 mV. Interpulse interval was 4 s. Right-hand trace shows current recruited by prepulse to 90 mV. *B*, six superimposed traces from the same cell as in *A* showing inactivation of currents induced by test pulses to 0 mV preceded by prepulses (400 ms) to −60 (zero prepulse), −50, −40, −30, −20 and −10 mV (not leak subtracted). Interpulse interval was 10 ms. *C*, plot of data from *A* and *B* showing voltage dependencies of potentiation (□) and inactivation (■) in this cell.

prepulse, 4 s delay, test pulse to 0 mV). Sixty-three per cent (19/30) of these cells expressed LVA VOCCs. Mean control current was 61·2 ± 10·0 pA ($n = 14$; range, 15–200 pA). All cells in which the LVA current was observed displayed significant (>20%) potentiation in response to prepulses to positive potentials. Voltage sensitivity was similar to potentiation of dexamethasone-treated cells and the mean response to a prepulse to +90 mV (Ba^{2+} saline) was 83 ± 23% ($P < 0·05$, paired t test, $n = 14$), similar to that seen in non-dexamethasone-treated cultures (n.s.).

DISCUSSION

Bone marrow stromal cells express a LVA VOCC which has biophysical characteristics similar to the neuronal T-current, is blocked by Ni^{2+} and DPH at concentrations similar to (though slightly higher than) those at which it blocks neuronal T-currents (e.g. Twombly, Yoshii & Narahashi, 1988; Gu & Spitzer, 1993) and is insensitive to dihydropyridines (Publicover *et al.* 1994). Repeated LVA current activation or application of depolarizing prepulses caused a clear potentiation of recorded current, resulting in an increase in current amplitude of up to 250%. The threshold for induction of this effect in saline with physiological concentrations of divalent cations was around −45 mV, and saturation occurred with prepulses to approximately +60 mV. Increasing prepulse potential beyond that required for saturation of potentiation (and well beyond the equilibrium potential for Ca^{2+} flux estimated from the data illustrated in Fig. 1) caused no modification of the response, indicating that potentiation is a voltage- rather than current-dependent process. Furthermore, potentiation was not dependent upon the presence or amplitudes of other currents.

Nature of the recruited LVA current

We do not believe that potentiation could reflect effects of prepulses on other currents, such as inhibition of an outward current carried by K^+ or Cl^- with rapid activation–inactivation kinetics, for the following reasons. (i) Potentiation occurs when K^+ channels are blocked both by intracellular Cs^+ and extracellular Ba^{2+}. (ii) $[Cl^-]_o$ is greatly enhanced in Ba^{2+} saline compared with Ca^{2+}–Mg^{2+} saline but this change does not modify the amplitude of potentiation. (iii) The voltage-induced current has an I–V relationship and kinetics very similar to those of the unpotentiated LVA VOCC. The time constant of current decay was 25% longer for control than recruited currents and the I–V relationships of control and recruited currents diverged at

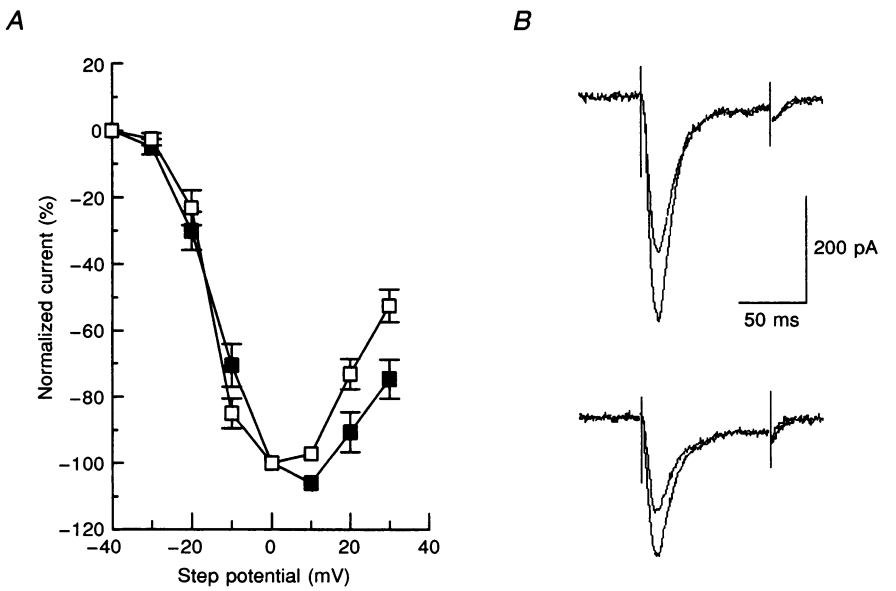

Figure 7. Characteristics of recruited current

A, mean current–voltage relationships (bars, ± S.E.M.) of control (■) and recruited (□) LVA currents from 4 cells. LVA currents were evoked by test pulses to −40, −30, −20, −10, 0, 10, 20 and 30 mV ($V_h = -60$ mV, Ba^{2+} saline) with and without preceding test pulses (400 ms, +30 mV, interpulse interval = 4 s). Recruited currents were obtained by subtraction of control from potentiated currents. Current amplitudes were normalized to that induced at 0 mV (HVA current threshold) before calculating means. *B*, effect of Ni^{2+}. Top panel shows superimposed control and potentiated currents from a cell bathed in Ba^{2+} saline ($V_h = -60$ mV, test pulse to 0 mV, 400 ms prepulse to +100 mV, interpulse interval = 4 s). Lower panel shows currents from the same cell, elicited under similar conditions but in the presence of 60 μM $NiCl_2$. Ni^{2+} inhibited control and recruited currents similarly.

positive potentials (Ba^{2+} saline). However, both of these differences are consistent with slight HVA current contamination of control current records. (iv) The sensitivities of the recruited current to inhibition by Ni^{2+} and DPH are identical to those of the unpotentiated current. (v) No outward currents were ever observed that might contribute to such a voltage-induced effect. The only outward current observed had a threshold at approximately +20 mV (Ba^{2+} saline) and was slowly activating and non-inactivating (Publicover *et al.* 1994)

We therefore suggest that the enhanced inward current reflects an increase in LVA Ca^{2+} current. The only previous description of voltage-induced potentiation of LVA VOCCs concerned potentiation of T-currents of coronary artery myocytes. Potentiation in these cells had a voltage dependency identical to that of steady-state inactivation and was associated with a marked change in current kinetics such that there was an increase in peak current but a 5% net decrease in charge transfer. Potentiation of the current was attributed to a voltage-induced alteration in the first latency of opening during recovery from inactivation (Ganitkevich & Isenberg, 1991). However, in our recordings there were no similarities between the voltage dependencies of potentiation and inactivation, both the threshold and slopes of the voltage relationships being different (Fig. 6). Furthermore, the kinetics of control and induced currents were strikingly similar such that potentiation clearly enhanced charge transfer (Fig. 3). The data described here are therefore not compatible with such a model and we conclude that they reflect a genuine recruitment of LVA VOCC by depolarizing voltage pulses.

In previous studies it has been shown that voltage-induced potentiation of HVA currents can occur in various ways, including a shift in gating mode, recruitment of a population of quiescent channels and a negative shift in channel activation voltage (Lee, 1987; Pietrobon & Hess, 1990; Artalejo, Rossie, Perlman & Fox, 1992; Sculptoreanu *et al.* 1993). Our data are not consistent with a shift in activation voltage, but a change in channel characteristics or a recruitment of silent channels could underlie our observations. Since we have not been able to establish any clear difference between the control and recruited currents, it is possible that these two currents are derived from the same population of channels. If this is the case then cell-to-cell variation in control current may reflect the proportion

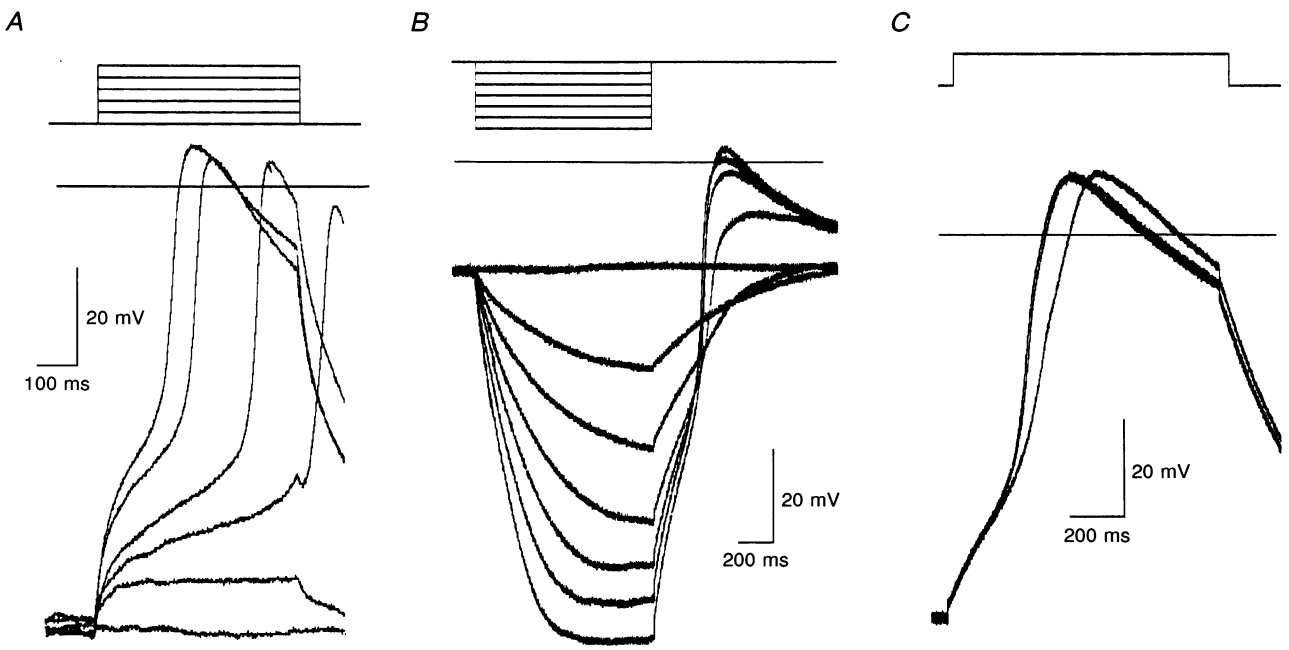

Figure 8. Behaviour of cells under current clamp

All recordings were carried out in Na^{+}–Ca^{2+}–Mg^{2+} saline containing 400 nM TTX. Pipettes contained K^{+} backfill. Horizontal lines in all three traces indicate 0 mV. *A*, induction of regenerative responses upon injection of depolarizing current. Cell was held at −90 mV by current injection (resting potential was −55 mV) and subjected to a series of depolarizing current injections (0, 50, 100, 150, 200, 250 pA) of 500 ms duration. *B*, regenerative responses induced upon relaxation of membrane potential after 1000 ms hyperpolarizing current injections. Resting potential was −23 mV, current injections were 0, −20, −40, −60, −80, −100, and −120 pA. *C*, reduced latency of regenerative response upon repeated activation. Responses were induced by 1 s, 60 pA current injections applied at 0·33 Hz. The first 3 responses are shown. Cell was held at −90 mV by injection of hyperpolarizing current. Same cell as in *B*.

of LVA channels that are available for activation without application of a prepulse. A consequent inverse variation in the proportion of channels available for recruitment would result in a negative relationship between control and induced current amplitudes. Such a relationship was not observed.

Processes underlying current recruitment

The data described here provide only limited information on the mechanism by which potentiation is induced. Recruitment of current occurred slowly (nearly 1000 ms for saturation at +10 mV in Ba^{2+} saline) and decayed over a period of up to 90 s. A voltage-induced, second messenger-mediated modulation or recruitment of channels is therefore a possibility, but we have so far found no evidence for involvement of ATP, GTP or kinases. Potential second messenger-independent mechanisms include a temporary relief of channel blockade, which is reimposed over a period of 10–90 s, or a direct shift of the channels to a higher conductance state, from which they decay with the kinetics observed in our recordings. If channel unblocking is involved, the blocking molecule is likely to be tethered, possibly being a part of the channel itself, since it is clearly not removed by cellular dialysis.

Physiological significance

Since LVA currents are unlikely to permit sufficient Ca^{2+} influx to cause a significant elevation of $[Ca^{2+}]_i$, the most significant effect of the phenomenon described in this report is probably to increase cell excitability in response to repeated activation, even at very low frequencies. During repetitive activity positive feedback can occur, LVA current potentiation enhancing further LVA current recruitment and facilitating the activation of other voltage-sensitive conductances. Such an effect could occur in various cell types in which rhythmic activation of T-currents occurs, the potentiating frequency band being set by the decay kinetics of T-current inactivation and recruitment.

Our current-clamp records suggest that LVA current potentiation acts in this way in bone marrow stromal cells. Approximately 50% of dexamethasone-treated stromal cells, like calvarial cells (Chesnoy-Marchais & Fritsch, 1988), generated slow action potentials. Action potential peaks were prolonged but rarely exceeded 5–10 mV (Fig. 8), possibly due to activation of a sustained outward current at approximately 0 mV (Publicover *et al.* 1994). The proportion of excitable cells in non-dexamethasone-treated preparations is liable to be lower due to the smaller LVA currents in these cells. Stromal cells express no detectable TTX-sensitive Na^+ current and the spikes were not inhibited by 200 nM TTX, sufficient to abolish Na^+ spikes in osteoblasts. We therefore conclude that the spikes are carried by LVA VOCCs (Chesnoy-Marchais & Fritsch, 1988). Repeated depolarizing pulses, delivered at 0·33 Hz, slightly increased spike amplitude and markedly reduced latency, indicating that the cells become more excitable upon repeated activation. A likely effect of LVA current recruitment in stromal cells is an enhanced activation of HVA currents and consequent Ca^{2+} influx, similar to T-current-dependent Ca^{2+} transients in embryonic neurons (Gu & Spitzer, 1993).

One way in which repeated LVA current activation may be induced is through low frequency, rhythmic induction of stretch-activated currents during locomotion (and consequent straining of the bone). Various stretch-activated channels have been shown to exist in bone cell lines (Duncan & Misler, 1989; Davidson *et al.* 1990; Duncan *et al.* 1992; Davidson, 1993) and are also present in marrow stromal cells (S. Publicover, unpublished data). LVA current spikes and consequent recruitment of HVA channels could occur either due to activation of depolarizing stretch-activated channels, or upon relaxation from hyperpolarizing potentials. Induction of calcium incorporation in ROS 17/2.8 cell cultures by cyclical loading at 0·05 Hz is blocked by verapamil (Vadiakis & Banes, 1992), indicating that HVA VOCCs may be involved in transduction of this mechanical stimulus. Similar studies on cultured marrow stromal cells have shown that loading of cultures induces expression of osteoblastic marker proteins and that this effect is blocked by verapamil (G. Thomas, L. Walker, A. El Haj & S. Publicover, in preparation).

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