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PHOSPHORYLATION RESTORES ACTIVITY OF L-TYPE CALCIUM CHANNELS AFTER RUNDOWN IN INSIDE-OUT PATCHES FROM RABBIT CARDIAC CELLS

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SUMMARY

1. Rundown of L-type calcium channels was studied in inside-out patches made from single isolated rabbit ventricular myocytes, using barium as the charge carrier.

2. In the cell-attached patches single-channel activity was stable for more than 15 min after the patch pipette sealed. β-Receptor stimulation by isoprenaline caused a characteristic increase in opening probability and the appearance of prolonged openings. When the patch was excised to the inside-out configuration and exposed to a simple ionic solution, channel activity disappeared within 1–2 min and never reappeared spontaneously.

3. After rundown of L-type channel activity in the excised patch, exposure of the inside face of the patch to MgATP and the catalytic subunit of the cyclic AMP-dependent protein kinase (PKAc) resulted in recovery of Ca^{2+} channel activity. Under these conditions channel activity could be even greater than under control cell-attached conditions, resembling channel activity after exposure to isoprenaline. This recovery of activity persisted many minutes, usually until the patch was lost. Addition of MgATP alone caused a small transient increase in channel activity in some patches.

4. Recovery of activity by MgATP and PKAc could be prevented by prior exposure of the excised patch to protein kinase inhibitor (PKI), or it could be abruptly terminated by exposure to PKI after recovery of activity. Addition to the pipette solution of okadaic acid, a protein phosphatase inhibitor, greatly slowed rundown. These findings support the proposal that dephosphorylation is an important component of rundown, and that phosphorylation is needed for channel opening activity.

5. Single-channel conductance was not altered by patch excision, but it was reduced after exposure of the excised patch to MgATP and PKAc. Mg^{2+} was responsible for this effect, probably by direct channel block from the inside, and Mg^{2+} also caused a negative shift in the channel activation, as expected from shielding of inside fixed negative charges.

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INTRODUCTION

Modulation of calcium current by the β-adrenergic–cyclic AMP cascade was first demonstrated in cardiac muscle, where it is a major regulator of cardiac cell function (Reuter, 1974). Extensive subsequent studies have shown that the L-type calcium channel is modulated in the heart and in many other cells by phosphorylation of the channel or a related protein through the enzymatic action of a cyclic AMP-dependent protein kinase (PKA) (See Pelzer, Pelzer & McDonald, 1990 for a review).

Although phosphorylation increases the cardiac L-type calcium current, a basal level of current is present in intact cells in the absence of exogenous stimulation of cyclic AMP production. While this may be the result of stimulation of phosphorylation by endogenous levels of cyclic AMP, dialysis of cells with an oligopeptide inhibitor of PKA (PKI) or with high levels of the regulatory subunit of PKA (to complex with any free catalytic subunit) reduced calcium current only 20–30% (Kameyama, Hescheler, Hofmann & Trautwein, 1986). In addition, cardiac calcium channels can be incorporated into planar bilayers, where they can show opening behaviour in the presumed absence of phosphorylation (Rosenberg, Hess, Reeves, Smilowitz & Tsien, 1986). Consequently, it has been assumed that cardiac calcium channels have normal opening probabilities in the absence of phosphorylation, but that their kinetic properties are modulated by the presence of a phosphate group.

In conflict with only a modulatory role for phosphorylation are experiments with whole-cell dialysis and with inside-out patches. Whole cardiac cell calcium current recorded with patch pipettes shows 'rundown', which is a spontaneous decrease in voltage-activated current after onset of dialysis by the pipette solution (Irisawa & Kokubun, 1983). Rundown is thought to be the result of washout of important cytoplasmic constituents or loss of intracellular calcium buffers. A phenomenon resembling rundown occurs quickly upon excision of a membrane patch containing calcium channels to expose the inside face of the membrane to simple salt solutions (Cavalié, Ochi, Pelzer & Trautwein, 1983). Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown (1987) have reported that at least part of the inside-out patch rundown can be delayed by inclusion in the solution exposed to the inside face of the membrane of agents to promote phosphorylation. The role of phosphorylation in rundown of calcium currents has also been investigated in non-cardiac cells. Armstrong & Eckert (1987) produced rapid loss of calcium channel activity by excision of patches from GH_3 cells and showed that this activity could be recovered by exposure to MgATP and PKAc. They proposed '... that dephosphorylation ..., not voltage, inactivates the channel and produces an irreversible loss of activity in the absence of rephosphorylation'.

In order to determine whether the proposal of Armstrong & Eckert (1987) is valid for cardiac L-type calcium channels, we examined the effects of adding components of the phosphorylation system to excised patches from rabbit ventricular myocytes. Addition of MgATP and PKAc resulted in recovery of cardiac calcium channel activity after initial rundown had occurred, similar to the results of Armstrong & Eckert (1987). This effect could be prevented or reversed by addition of an inhibitor of protein kinase A. Block of protein phosphatase before excision greatly slowed

rundown. These results imply that phosphorylation is necessary for maintenance of normal channel function and that channel open probability is very low in the absence of phosphorylation. We also found that increased intracellular Mg^{2+} reduced single-calcium channel conductance, presumably by open channel block, and shifted the channel kinetics in the negative direction, as expected from shielding of fixed negative intracellular surface charges. A preliminary account of part of this work has been presented in abstract form (Ono, January & Fozzard, 1990).

METHODS

Cell isolation procedure

Myocytes were isolated from rabbit hearts by enzymatic treatment and mechanical dispersion (Poole, Halestrap, Price & Levi, 1989). The rabbits (2–3 kg) were anaesthetized with Ketamine HCl (80–100 mg kg^{-1}) and Xylazine HCl (3–5 mg kg^{-1}) prior to excision of the hearts. The hearts were retrogradely perfused via the aorta at a constant flow rate of 5–7 ml min^{-1}. The perfusion buffer contained (mM): 130 NaCl, 5 HEPES, 10 glucose, 20 taurine, 10 creatine, 5·4 KCl, 3·5 $MgCl_2$ and 0·4 NaH_2PO_4 in ultra-pure double processed water (Milli Q instrument from Millipore Corp., USA), neutralized to pH 7·25 with NaOH and gassed with 100 % O_2 (solution A). The heart was perfused at 37 °C with solution A + 0·75 mM-$CaCl_2$ for 4 min, and then for 4 min with solution A + 0·1 mM-ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA). Finally the heart was perfused with solution A + 0·08 mM-$CaCl_2$, 1 mg ml^{-1} collagenase (Worthington, USA; type II) and 0·1 mg ml^{-1} protease (Sigma, USA; type 14) for 10 min. The digested heart was removed, chopped coarsely and shaken for four 5 min periods in a plastic flask containing the enzyme solution with the addition of 1 % (w/v) bovine serum albumin. After each 5 min incubation the tissue was filtered though a nylon gauze (pore size 200 μm), and undigested material shaken for a further period. The isolated cells filtering through the gauze were collected by centrifugation at low speed for 120 s. The supernatant was then changed to a solution containing 150 mM-potassium glutamate and 10 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (pH 7·2). Cells were then stored in a solution containing 130 mM-potassium glutamate, 5·7 mM-$MgCl_2$, 0·1 mM-EGTA, and 10 mM-HEPES (pH = 6·9). The preparation routinely yielded 50–90 % Ca^{2+}-tolerant cardiac myocytes with rod-shaped morphology. Small aliquots of cells were added to the bath solution (see below for contents) in a perfusion chamber of 200 μl volume constructed on a glass cover-slip and mounted on a stage of an inverted microscope (Diaphot, Nikon, Japan).

Electrical recording

Single-channel recordings were made using the cell-attached and inside-out mode patch-clamp method of Hamill, Marty, Neher, Sakmann & Sigworth (1981). Glass suction pipettes were fabricated from haematocrit capillary tubes (Drummond Scientific, USA). Pipettes were pulled with a multi-stage micropipette puller (Sutter Instrument Co., USA; Model P-87), coated with Sylgard 184 (Corning Medical, USA), and heat-polished to a final tip diameter of 1·5–3·0 μm using a microforge (Narishige, Japan; Model MF-83). When filled with pipette solutions (see below), the pipettes had resistances of 0·5–1·2 MΩ. Patches contained typically one to three channels, judged from the maximal number of overlaps of openings in the entire record. This estimated number of channels was used for the calculation of open probability (NP_o). The bath solution was connected to ground via a 150 mM-KCl–agar bridge and a Ag–AgCl half-cell electrode. Electrical contact with the pipette solution was via a chlorided silver wire. The electrode potential was adjusted to give a zero current between the pipette solution and the bath solution immediately before attempting to make a patch. Gigaohm seals between the pipette and the single ventricular cells were obtained by applying gentle suction to the pipette after contacting the cell membrane and the suction was released after the gigaohm seal formation to prevent hyper-concavity of the patch membrane in the glass pipette. Seal resistances for these experiments ranged between 20 and 120 GΩ. Single-channel currents were recorded in the cell-attached patch and inside-out patch configuration using a patch-clamp amplifier (EPC-7, List, FRG) and low-pass filtering at 2 kHz. The inside-out patches were obtained by briefly (< 1 s) passing the pipette tip through the air–water interface after the

formation of the membrane vesicles with the pipette tips as described by Hamill *et al.* (1981). The experiments were performed at room temperature (20–22 °C).

Data analysis

The capacitive transient was partially compensated by analog circuitry, and the residual transient was removed by subtracting the average current from equipotential steps with no channel openings. The baseline also was adjusted for each sweep so that current averaged 0 pA when channels were closed. An amplitude histogram was constructed from the corrected traces. The open channel amplitude was estimated by the peak current or by the maximal value of a Gaussian function fit. An opening or closing transition was identified by the presence of two successive data points above or below the 50% amplitude level. Double openings were rare (< 3%; see Fig. 3), and for them the subsequent closures were assigned randomly to the openings.

Solutions

The bathing solution contained (mM): 140 potassium aspartate and 10 HEPES (pH adjusted to 7·4 with KOH). This solution depolarized the ventricular cells to approximately 0 mV. The internal solution for the suction pipette contained (mM): 110 $BaCl_2$, 30 TEA-Cl, 0·05 tetrodotoxin (TTX), and 10 HEPES (pH adjusted to 7·4 with tetraethylammonium hydroxide (TEA-OH). TEA^+ and TTX were added to block K^+ and Na^+ channels, respectively. The agents used in the experiments were as follows: isoprenaline, 3 and 15 μM; magnesium adenosine 5′-triphosphate (MgATP) 1·8 and 7·2 mM; magnesium aspartate 2·5 and 10 mM; the catalytic subunit of cyclic AMP-dependent protein kinase (PKAc) 2·2 and 8·8 units ml^{-1} (U ml^{-1}); protein kinase inhibitor (PKI), 0·23 mM. PKAc was activated with 200 mM-dithiothreitol (DTT) for 90 min and diluted with the bath solution plus 2 mM-DTT. These reagents were obtained from Sigma Chemical Co. Okadaic acid, a generous gift from Dr Y. Tsukitani (Fujisawa Pharmaceutical Co., Japan), was stored as 10 mM with 100% ethanol and was added to the pipette solution to make a concentration of 5 μM just before the experiment. When used, Bay K 8644 (5 μM, Miles Pharmaceutical) was added to the pipette solution (from a 10 mM stock solution of Bay K 8644 dissolved in 100% ethanol). Where appropriate, data are reported as means ± standard deviation.

RESULTS

Stability of channel behaviour in the cell-attached mode

In the cell-attached recording mode no alteration in the cytoplasm is expected; consequently, rundown should not occur. A diary of NP_0 (proportion of the activating step time that the channels were open) during 195 ms duration steps from −70 to 0 mV in a cell-attached patch with two channels (Fig. 1) shows values between 0 (null sweeps) and 0·2, with sweep-to-sweep variation in activity. This pattern was stable for at least 15 min. The ratio of average NP_0 during the first 3 min period relative to the last 3 min period was 1·09 for this patch, and it was 0·96 ± 0·08 for a population of five cells. While the pattern appeared to show periodicity of activity, a histogram of individual sweep NP_0 values was well fitted by a single exponential, as expected for stationary kinetics. The open time distributions under these conditions were fitted by single exponential curves. Openings early in the epoch had mean open times of 0·32 ms and this value did not change when successive epochs were analysed. In addition, single-channel current early after establishment of the seal was −1·45 pA at 0 mV and did not change during the recording period. Similar stability of open times and single-channel currents were obtained during prolonged recording from thirteen other cells (mean open time 0·33 ± 0·02 ms). Consequently, no rundown or change in kinetics was apparent during the cell-attached recording period.

Exposure of the cell to isoprenaline produced a characteristic change in single-

channel open durations previously described (e.g. Reuter, Stevens, Tsien & Yellen, 1982) and called mode 2 behaviour by Yue, Herzig & Marban (1990). Modest increase in NP_o product and reduction of null sweeps could be seen with 3 μM-isoprenaline. With 15 μM-isoprenaline the NP_o product was greatly increased and prolonged

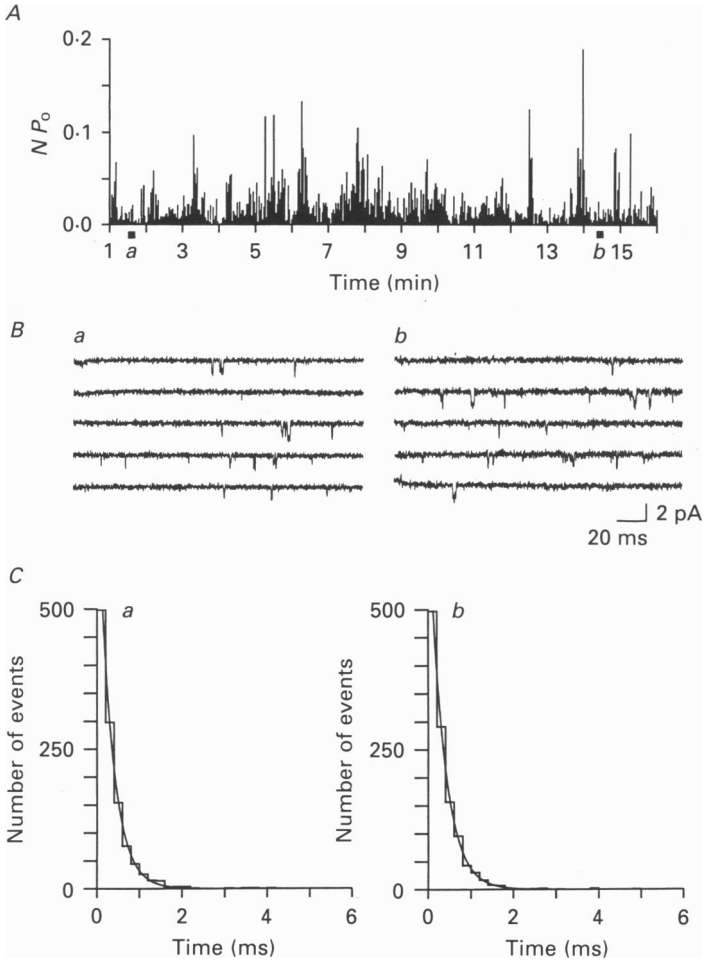

Fig. 1. Stable calcium channel activity during prolonged recording from a cell-attached patch with two channels. *A*, diary of NP_o for 0·5 Hz steps of 195 ms in duration from −70 to 0 mV. *B*, examples of sequential individual sweeps recorded early (*a*) and late (*b*), shown by ■ in the diary in panel *A*. In these records the voltage steps began 5 ms after beginning of the sweep. *C*, open duration histograms from the first 4 min epoch (*a*) and the last 4 min epoch (*b*). The data were fitted by single exponential curves with rate constants of 0·32 and 0·34 ms. The first bins (0·0–0·2 ms) were excluded for fitting of the exponential functions.

openings began to appear (Fig. 2). Open duration histograms showed the appearance of a second population of long openings. Figure 3 illustrates open time distributions for the patch shown in Fig. 2. Measurements in the cell-attached mode yielded a distribution well fitted by a single exponential function with a rate constant of

0·34 ms. After exposure to 15 μM-isoprenaline the distribution was best fitted by two exponentials, one with the same rate constant seen in the control distribution and the other with a rate of constant of 1·01 ms. Similar results were seen in nine experiments with 15 μM-isoprenaline (fast rate constant, $0{\cdot}32 \pm 0{\cdot}02$ ms; slow rate constant, $1{\cdot}07 \pm 0{\cdot}22$ ms).

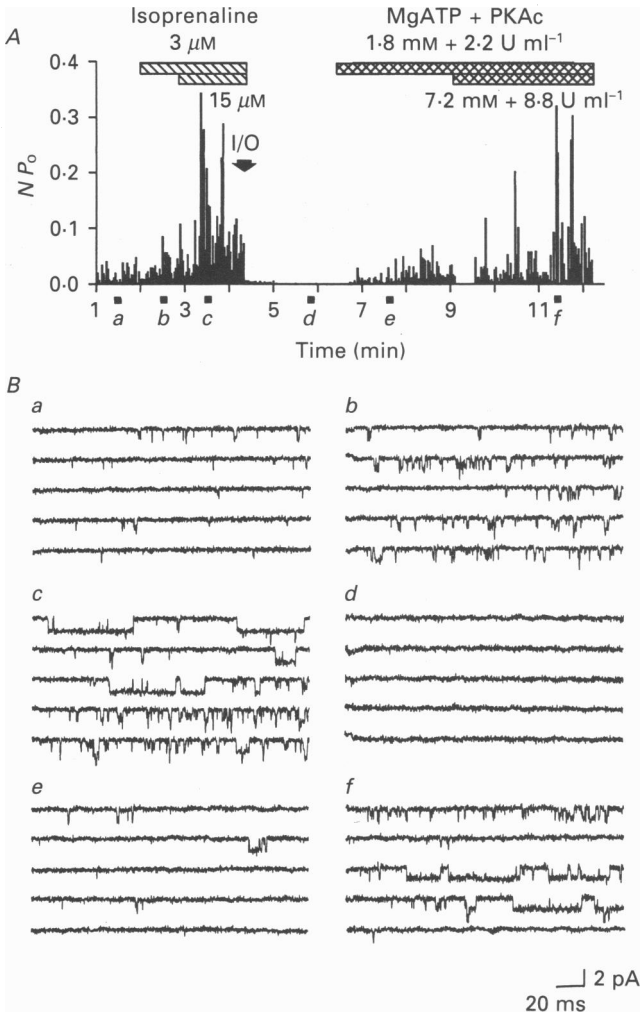

Fig. 2. Response to excision of a patch treated with isoprenaline and to addition of MgATP and PKAc to the inside solution. *A*, diary of NP_o. Above the plot are noted the composition and timing of additions to the inside bathing solution. The arrow labelled I/O is the time of patch excision. *B*, sequential epochs (*a–f*) of five consecutive sweeps recorded at the times indicated by ■ in panel *A*. In these records the voltage steps began 5 ms after beginning of the sweep: *a*, control cell-attached recording; *b*, during the application of 3 μM-isoprenaline; *c*, 15 μM-isoprenaline; *d*, 90 s after excision of the patch; *e*, during the application of 1·8 mM-MgATP + 2·2 U ml^{-1} PKAc; and *f*, 7·2 mM-MgATP + 8·8 U ml^{-1} PKAc. Shortly after application of 7·2 mM-MgATP + 8·8 U ml^{-1} PKAc the current traces were lost for technical reasons.

Rundown upon excision of the patch into the inside-out mode

After recording of channel activity in the cell-attached mode, the patch was excised into the inside-out configuration, where it was exposed to a buffered potassium aspartate solution. Channel activity rapidly diminished to low levels

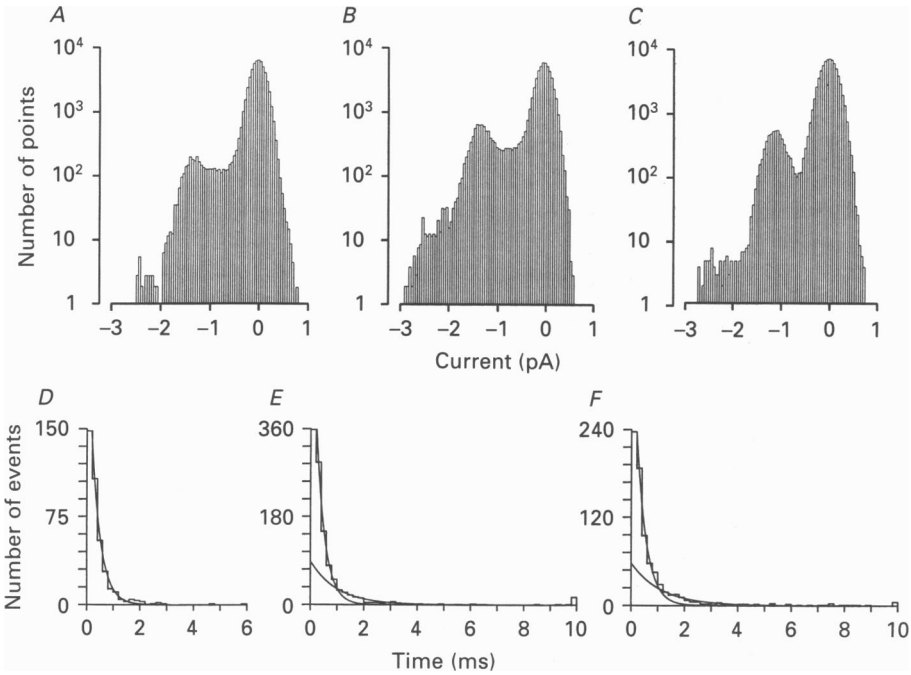

Fig. 3. Single-channel properties with isoprenaline and with addition of MgATP and PKAc. The upper row (*A–C*) are amplitude histograms in the control cell-attached mode (*A*), after addition of 15 μM-isoprenaline to the cell bathing solution (*B*), and after excision to the inside-out mode and restoration of channel activity with 7·2 mM-MgATP and 8·8 U ml^{-1} PKAc (*C*). Measurements of single-channel currents (at 0 mV) were −1·43 pA (*A*), −1·43 pA (*B*), and −1·21 pA (*C*). The lower row (*D–E*) are open duration histograms from the three conditions shown in the upper row. After exclusion of the first 0·2 ms the data were fitted by a single exponential function with a rate constant of 0·34 ms in control (*D*), and by a double exponential function with fast and slow rate constants of 0·33 ms and 1·01 ms with 15 μM-isoprenaline (*E*). After restoration of activity in the inside-out patch by 7·2 mM-MgATP and 8·8 U ml^{-1} PKAc the double exponential fit showed similar rate constants to those after isoprenaline (0·37 and 1·14 ms).

within a minute. Comparison of NP_0 before excision to that between 2 and 3 min after excision showed that the average activity had fallen to 1·4 % of its pre-excision value ($n = 24$), and no patch showed more than 5 % of its former NP_0 value by 2–3 min.

Recovery of channel activity by MgATP and PKAc

Simultaneous addition of 1·8 mM-MgATP and 2·2 U ml^{-1} PKAc to the intracellular bathing solution 2 min after patch excision, when no activity remained, resulted in a slow recovery of channel activity to levels similar to normal cell-attached patches (Fig. 2). Addition of a higher level of 7·2 mM-MgATP and 8·8 U ml^{-1} PKAc increased

channel activity to a higher level and with the long open times characteristic of the effects of isoprenaline. Figure 3*F* illustrates the two-exponential character of the recovered channel open duration distribution, with a faster rate constant (0·37 ms) similar to the untreated channels and a slower one (1·14 ms) similar to the second rate

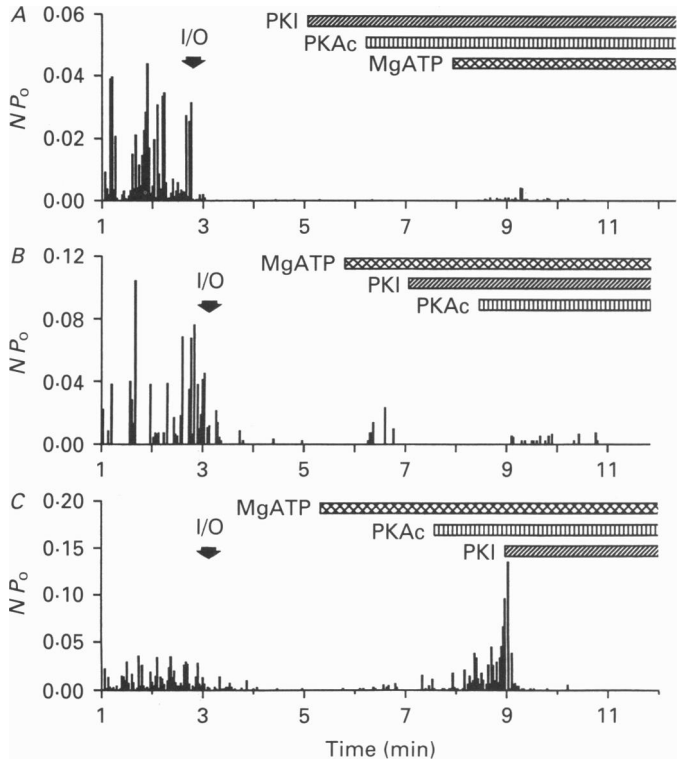

Fig. 4. Effect of PKI on the MgATP–PKAc effect. Three separate experiments are illustrated. *A*, 0·23 mM-PKI added before 5 mM-MgATP and 8·8 U ml^{-1} PKAc. *B*, PKI added between MgATP and PKAc. *C*, PKI added after MgATP and PKAc. Test depolarizations were made at 0·5 Hz.

constant after isoprenaline. Similar open time distributions were seen in seven patches after treatment with MgATP and PKAc (fast rate constant, 0·36 ± 0·02 ms; slow rate constant, 0·99 ± 0·23 ms). MgATP and PKAc could be added simultaneously or sequentially, and the order of addition was not important. However, if the excised patch was not treated with MgATP and PKAc for 10 min after complete rundown, recovery was not achieved ($n = 3$). No exact measure of the duration of the effects of MgATP and PKAc could be made because the patches were lost before rundown was seen. The longest maintenance of activity was in two patches that lasted for 7 min, suggesting that the MgATP and PKAc addition may have increased patch instability. A small transient increase in channel activity occurred in four patches out of nine trials after addition of only MgATP to the intracellular solution, but the response never exceeded 10% of the pre-excision level of activity.

If the recovery after MgATP and PKAc was the result of phosphorylation, then it should be prevented or reversed by PKI. Figure 4 shows that recovery of channel activity was prevented by addition of a high concentration of PKI (0·23 mM) before MgATP and PKAc. Channel activity after MgATP and PKAc in the presence of PKI

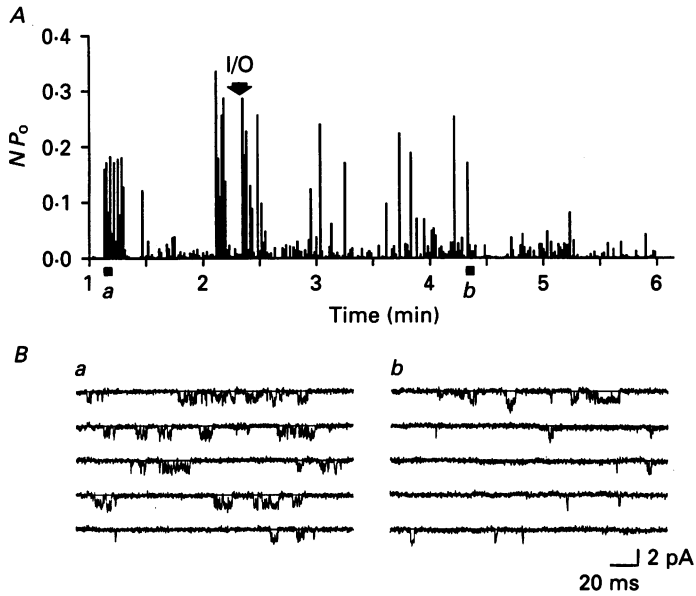

Fig. 5. Effect of okadaic acid on rundown after patch excision. Diary of NP_o before and after patch excision in the presence of 5 μM-okadaic acid in the pipette solution. Five sequential sweeps are illustrated from the identified epochs in panel *A*. Steps were made at the rate of 1·0 Hz. In these records the voltage step began 5 ms after beginning of the sweep.

was 3·1 ± 2·8 % of control activity ($n = 7$). Similar results were obtained when PKI was given after MgATP but before PKAc ($n = 6$). If PKI were added after recovery had occurred, it terminated the activity rapidly. The effects of MgATP and PKAc were prevented in all of thirteen experiments and reversed in all of three experiments.

Delay of rundown by inhibition of protein phosphatase

If rundown was the result of dephosphorylation during the first few minutes after excision of the patch, then inhibition of the protein phosphatase should slow the rundown process. Okadaic acid (5 μM) was added to the pipette solution in order to inhibit the phosphatase activity, and channel activity was compared before and after patch excision (Fig. 5). In the illustrated experiment the NP_o product was maintained for at least 3 min following excision. Before excision of this patch the average NP_o product was $4{\cdot}19 \times 10^{-2}$ (79 sweeps). During the first 60 s after excision the activity was 98 % of pre-excision level, but it declined to 37 % during the third minute. Similar experiments with six patches demonstrated activity of 25 ± 18·5 % in the

third minute after excision. As noted before, rundown in simple salt solution showed activity of only 1·4% in the third minute. Consequently, inhibition of protein phosphatase slowed but did not prevent rundown.

Effect of excision in single-channel conductance

After recovery of channel activity by MgATP and PKAc single-channel currents at 0 mV were slightly reduced (Fig. 6). The slopes of single-channel i–V curves

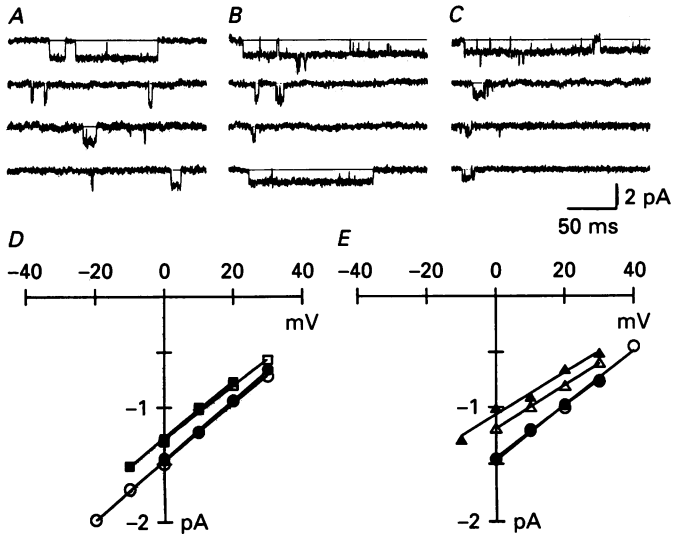

Fig. 6. Effect of excision and addition of MgATP, PKAc, or magnesium aspartate on single channels. Upper row (A–C) illustrates individual sweeps of channel currents upon steps from −70 to 0 mV (A), +10 mV (B), and +20 mV (C) for recordings made in the cell-attached mode (upper), after excision to the inside-out mode (second from top), after addition of 10 mM-MgATP (second from bottom) and after further addition of PKAc (bottom). The voltage step began 5 ms after beginning of the sweep. Panel D shows i–V relationships from amplitude histograms of data like those seen in the upper panels for control cell-attached patch (○), inside-out patch (●), application of 10 mM-MgATP (□), and addition of 8·8 U ml^{-1} PKAc (■). Panel E shows i–V relationships in control cell-attached patch (○), inside-out patch (●), addition of 2·5 mM-magnesium aspartate (△), and addition of 10 mM-magnesium aspartate (▲) into the solution bathing the inside face of the membrane.

measured in cell-attached patches showed a conductance of 25·2 ± 1·0 pS (n = 11). After excision the conductance was unchanged at 25·0 ± 1·8 pS (n = 11). Addition of MgATP reduced single-channel conductance in six of seven experiments. The average conductance after addition of MgATP was 23·6 ± 2·4 pS ($P < 0·08$; n = 7). Finally, no further change in single-channel conductance occurred upon addition of PKAc (24 ± 1·7 pS; n = 4).

The basis of the change in single-channel conductance upon addition of MgATP was examined in separate experiments, in which Bay K 8644 (5 μM) was added to prolong openings and thereby to enhance the accuracy of the conductance measurement. Bay K 8644 itself had little effect on the pattern of rundown or

recovery. The *i–V* relationship was first measured in cell-attached patches and immediately after excision. Magnesium aspartate at 2·5 mM was then added, followed by 10 mM-magnesium aspartate. No change in the single-channel conductance was seen upon excision, but addition of magnesium aspartate reduced the conductances progressively ($n = 3$).

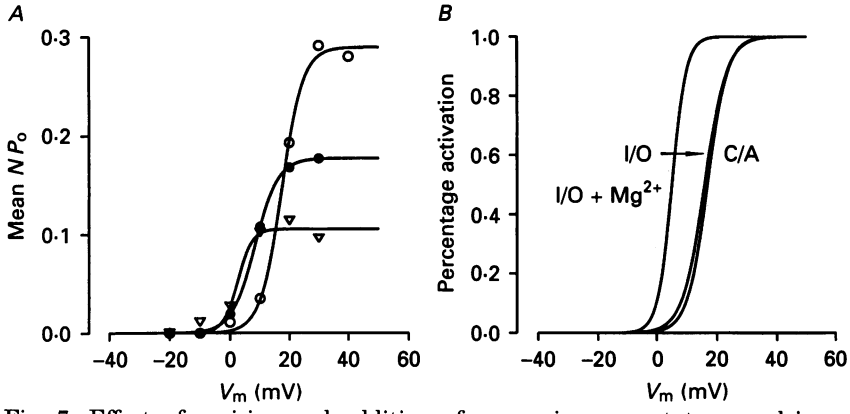

Fig. 7. Effect of excision and addition of magnesium aspartate on calcium channel activation. *A*, cell-attached mode NP_o at different activating voltages during 195 ms steps from −70 mV (○), after excision and exposure to 2·5 mM-magnesium aspartate (●), and after exposure to 10 mM-magnesium aspartate (▽). These observations were made in the presence of Bay K 8644 and progressive rundown. Each point represents the average of ten to fifteen sweeps. The half-activation voltages were +17·4, +8·3 and +2·5 mV. *B*, best-fit activation curves from three experiments normalized to maximal activation for cell-attached mode (C/A), inside-out mode (I/O), and 10 mM-magnesium aspartate (I/O + Mg^{2+}), with half-activation voltages of +16·8, +15·8 and +5·0 mV.

In order to investigate the effect of Mg^{2+} on channel kinetics, the NP_o product was measured as a function of the activating voltage step (Fig. 7). These experiments were made in the presence of Bay K 8644. Rundown was occurring during the observations. Half-activation for the control cell-attached patch was +17 mV, and after excision it was unchanged. Addition of 10 mM-magnesium aspartate shifted the activation relationship to +5 mV ($n = 3$).

DISCUSSION

Rundown of L-type Ca^{2+} current first became apparent when investigators began to use the relatively large-diameter patch pipette method for low resistance access to the cell interior (Kostyuk, Veselovsky & Fedulova, 1981; Irisawa & Kokubun, 1983; see Pelzer *et al.* 1990, for review). The phenomenon is important for two reasons: it implies that the cytoplasm is important for the function of calcium channels, and it complicates and frustrates experimental study of the Ca^{2+} current.

Several cytoplasmic processes are known to influence Ca^{2+} currents. Intracellular Ca^{2+} can inactivate the current (Brehm & Eckert, 1978) and several reports indicate that maintenance of low intracellular Ca^{2+} by buffering can slow rundown (Hagiwara & Byerly, 1981; Belles, Malécot, Hescheler & Trautwein, 1988). The mechanism of this effect of Ca^{2+} buffering may be in part avoidance of Ca^{2+}-dependent channel

inactivation, but also the Ca^{2+} channels or their modulatory proteins can be damaged by Ca^{2+}-activated proteases (Chad & Eckert, 1986). Recently, Romanin, Grösswagen & Schindler (1991) have reported that calpastatin, a blocker of calpain, slows rundown of channel activity in the inside-out patch.

The best-documented cytoplasmic system to modulate Ca^{2+} currents is enzymatic phosphorylation by the cyclic AMP-dependent protein kinase. Rundown might be expected if dialysis of the cytoplasm by a patch pipette solution reduced the cytoplasmic levels of cyclic AMP, PKA, ATP, or any other co-factor or modulator of this enzymatic cascade. Efforts to replace these components experimentally have led to conflicting results, with some investigators reporting slowing of rundown and others reporting no effect (Kostyuk *et al.* 1981; Irisawa & Kokubun, 1983; Lee & Tsien, 1984; Yatani *et al.* 1987; Hescheler, Mieskes, Rüegg, Takai & Trautwein, 1988). Explanations for the failure of experiments to be reproducible include the difficulty of delivery of large molecules through the small patch pipette tip and the consequent uncertainty of the intracellular concentration of the component, failure to replace all of the necessary components, and other uncontrolled factors causing rundown. Indeed, the rundown process has several kinetic phases in whole-cell studies (Belles *et al.* 1988), suggesting that there may be several overlapping mechanisms.

The most effective way to examine the role of the cytoplasm in maintenance of calcium channel function is to excise the patch so that its intracellular face may be bathed by artificial solutions with the ingredients proposed to be important in the rundown process. This approach also permits recording of the rundown phenomenon on the single calcium channel properties that could contribute to the decrease in cell current. Excision of the patch into the inside-out configuration and exposure to balanced salt solutions results in rapid disappearance of Ca^{2+} channel activity (Cavalié *et al.* 1983; Nilius, Hess, Lansman & Tsien, 1985; Yatani *et al.* 1987; Kaibara & Kameyama, 1988). Yatani *et al.* 1987 demonstrated reduced rundown in cardiac cells by addition to the solution bathing the inside face of the membrane of various components in the phosphorylation cascade, including cyclic AMP, PKAc, ATP and GTP, although they did not see recovery of activity once it had disappeared.

Phosphorylation slows rundown

In these experiments the recordings in cell-attached patches showed stable channel activity. However, within seconds of patch excision the activity decreased and became negligible within 2–3 min. After rundown was complete and all activity had disappeared, addition of MgATP and PKAc to the inside bathing solution promptly restored activity to its pre-excision level for a number of minutes. The duration of the restoration was difficult to ascertain because of loss of the patch seal after several minutes. Both MgATP and PKAc were required to recover activity.

The most plausible explanation for these results is that excision of the patch removed the soluble components of the phosphorylation cascade, and their restoration permitted rephosphorylation. Since a stable phosphorylation rate prior to excision requires a dynamic balance between phosphorylation and dephosphorylation, removal of the phosphorylation machinery permits membrane-associated phosphatases to dephosphorylate (Kume, Takai, Tokuno & Tomita, 1989),

abolishing channel activity rapidly. Addition of the missing components restores the balance, and activity returns.

A non-enzymatic action of MgATP and PKAc effect was excluded, since prior addition of the specific PKA inhibitor PKI could prevent restoration of activity, and subsequent addition of PKI could terminate activity. This is similar to the results of Kameyama *et al.* (1986), who found, using whole-cell currents, that PKI and the regulatory subunit of PKA could both reverse the isoprenaline-induced increase. However, in their experiments PKI did not abolish calcium currents.

Addition of MgATP alone to the inside-out patch after complete rundown sometimes stimulated a low level of calcium channel activity. It is possible that under the conditions of this experiment the first ingredient of the protein phosphorylation cascade to be depleted by excision was MgATP and the remaining PKA used the added substrate to phosphorylate the channels briefly, before PKA was also depleted.

Further support of an important role of phosphorylation in maintaining channel activity in the inside-out patch is found in the effect of okadaic acid, a potent inhibitor of types 2A and 1 protein phosphatases (Hescheler *et al.* 1988). Okadaic acid in the pipette solution substantially slowed rundown of channel activity, which suggests that the protein phosphatase remains associated with the patch after its excision. This result is also consistent with the finding that okadaic acid increased whole-cell Ca^{2+} current (Hescheler *et al.* 1988). Phosphatase inhibition by okadaic acid would be expected only to slow rundown, since washout removes the enzymatic system for rephosphorylation.

Although it is reasonable to conclude that these experiments demonstrate an important role of phosphorylation in maintaining kinetic function of Ca^{2+} channels in inside-out patches, we cannot provide direct evidence that it is the Ca^{2+} channel itself that is phosphorylated. The phosphorylated protein could be an associated component of the channel such as a subunit. Calcium channels are composed of several subunits, and the α_1- and β-subunits have phosphorylation sites (Catterall, 1988). Alternatively, the phosphorylated protein could be separated from the channel, but modulate it. Resolution of this question may require channel studies in reconstituted (Imoto, Yatani, Reeves, Codina, Birnbaumer & Brown, 1988) or expression systems (Lacerda, Kim, Ruth, Perez-Reyes, Flockerzi, Hofmann, Birnbaumer & Brown, 1991).

Extrapolation of this evidence for dephosphorylation in rundown in inside-out patches to rundown in whole-cell experiments is attractive. However, the report of Kameyama *et al.* (1986) that intracellular dialysis of PKI only partially reduced whole-cell Ca^{2+} current is inconsistent with that interpretation. It is possible that in their whole-cell experiments the concentration of PKI was insufficient to block PKA completely. Alternatively, the intact cell may have additional protein kinases that can maintain some level of phosphorylation in spite of inactivation of PKA. Indeed, it has been reported that protein kinase C action can increase calcium current transiently (Lacerda, Rampe & Brown, 1988).

An additional concern is that channel activity was submaximal in our cell-attached recordings. Addition of MgATP and PKAc to the inside-out patch might simply be enhancement of activity of a steadily diminishing number of functional

Ca^{2+} channels. If half the maximal activity was present in the cell-attached mode, and after excision we had maximal activity of half the number of channels, it would appear that full recovery had occurred. We sought to avoid this possibility by maximally enhancing channel activity by isoprenaline prior to patch excision. Precise determination of the number of channels is difficult because of the low probability of opening and the necessarily limited time of observation. We attempted to reduce any error in estimating channel number in the patch before and after excision by comparison during maximal activation with isoprenaline (15 μM) before and PKAc (8·8 U ml^{-1}) and MgATP (7·2 mM) after excision. No loss could be detected under these conditions. Isoprenaline at higher concentrations not only increased the number of sweeps with openings, but it also resulted in the appearance of many long openings. This was clearly demonstrated in the open time histograms, where a second population of long openings appeared. While this effect of catecholamine stimulation has been reported occasionally in the past, Yue *et al.* (1990) characterized it as 'mode 2' behaviour similar to that seen with Bay K 8644 (Nowycky, Fox & Tsien, 1985). Furthermore, the same behaviour was seen in these experiments on the inside-out patch after exposure to MgATP and PKAc, confirming the suggestion of Yue *et al.* (1990) that the change in channel kinetics is a consequence of phosphorylation.

Role of phosphorylation in Ca^{2+} channel activity

In these experiments loss of phosphorylation led to near abolition of channel activity, contrary to the usual view that the channel can gate normally in the absence of phosphorylation (Pelzer *et al.* 1990). Armstrong & Eckert (1987) proposed that dephosphorylation is intimately involved in each individual channel inactivation step. Although our results are consistent with their suggestion, they could also be explained by a switch to a mode in which the channels would access the open state only rarely. It is interesting to note that during rundown of whole-cell L-type cardiac calcium currents the channel putative gating charge does not change (Hadley & Lederer, 1991). Resolution of the mechanism of action of phosphorylation will require more precise measurements of the on–off rates of phosphorylation, which may be possible using the inside-out patch.

Effect of inside Mg^{2+} on Ca^{2+} channels

Exposure of the inside of the patch to millimolar concentrations of Mg^{2+} reduced the single-channel conductance. It is known that extracellular divalent ions can block sodium currents (Woodhull, 1973), and that outside calcium and magnesium reduce apparent sodium single-channel conductances by a rapid block–unblock interaction (Yamamoto, Yeh & Narahashi, 1984; Sheets, Scanley, Hanck, Makielski & Fozzard, 1987). Recently, Cukierman, Zinkand, French & Krueger (1988) showed a reduction in single sodium channel current by inside calcium, presumably also by rapid block–unblock. Such a rapid block–unblock process could explain the small fall in calcium channel currents seen in these experiments. Our i–V curves illustrating the effects of Mg^{2+} did not show voltage dependence, but the voltage range studied was small. Agus, Kelepouris, Dukes & Morad (1989) and Harzell & White (1989) have reported that increased inside Mg^{2+} can reduce whole-cell calcium current

substantially, and the effect on single-channel conductance demonstrated here may contribute to part of the overall effect of inside Mg^{2+}.

Inside Mg^{2+} also shifted the activation curve for single calcium channels in the negative direction. The well-known effect of divalent ions to shield or bind fixed surface charges, thereby altering the electric field at the channel voltage sensor, is sufficient to explain this effect of inside Mg^{2+}. Cukierman *et al.* (1988) reported a 7–14 mV hyperpolarizing shift for sodium channels in response to 7·5 mM inside Ca^{2+}. Hartzell & White reported a 10 mV hyperpolarizing shift of calcium channel activation in response to an increase from 0·3 to 3 mM inside Mg^{2+}, although Agus *et al.* (1989) failed to see a shift of calcium channel availability by 9·4 mM inside Mg^{2+}. Our results support the conclusion of Hartzell & White (1989) that fixed negative charges exist on the inside of the calcium channel.

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