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RUNDOWN OF *N*-METHYL-D-ASPARTATE CHANNELS DURING WHOLE-CELL RECORDING IN RAT HIPPOCAMPAL NEURONS: ROLE OF Ca^{2+} AND ATP

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SUMMARY

1. *N*-methyl-D-aspartate (NMDA) channel activity was studied on cultured rat hippocampal neurons in whole-cell voltage-clamp mode. NMDA responses were evoked by rapid application of NMDA and the cytosol was modified using pipette dialysis and intracellular perfusion.

2. In the presence of 2 mM $[Ca^{2+}]_o$ with 2·4 mM BAPTA (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid) and 0·4 mM Ca^{2+} in the whole-cell pipette, the response evoked by regular applications of 10 μM NMDA gradually decreased during prolonged whole-cell recording. After 25 min the peak current was reduced to $56 \pm 1{\cdot}6$% of control. Channel 'rundown' could be prevented by inclusion of an ATP regenerating solution in the pipette.

3. Rundown did not occur in Ca^{2+}-free medium even in the absence of added ATP regenerating solution. Rundown was also prevented by increasing $[BAPTA]_i$ to 10 mM whereas raising $[Ca^{2+}]_i$ by inhibiting the Na^+–Ca^{2+} exchanger or by perfusing the patch pipette with high $[Ca^{2+}]_i$ (15–1000 μM) reversibly inhibited the NMDA current. By contrast, the rundown of kainate responses was Ca^{2+}-independent.

4. The rate and reversibility of rundown was use-dependent. Rundown did not occur with infrequent NMDA applications (0·2/min). Following channel rundown in Ca^{2+}-containing medium, a 5 min pause in agonist applications or adding ATP regenerating solution by intracellular perfusion resulted in complete recovery. However, rundown did not recover following large currents evoked by 300 μM NMDA or when 10 mM EGTA was used as the intracellular buffer. Protease inhibitors did not prevent irreversible rundown.

5. ATP-γ-S (4 mM) was less effective than the ATP regenerating solution in preventing rundown. Likewise, intracellular dialysis with alkaline phosphatase, phosphatase 1 or calcineurin did not induce rundown and addition of phosphatase inhibitors also did not block rundown. Thus receptor dephosphorylation did not appear to be primarily responsible for channel rundown.

6. The mean open time and unitary conductance of the NMDA channel were unaffected by rundown as estimated by fluctuation analysis. The conductance was $42{\cdot}8 \pm 2{\cdot}9$ nS before and $43{\cdot}7 \pm 2{\cdot}8$ nS after rundown. The mean open times were 17·3

and 4·0 ms before and 15·9 and 4·0 ms after rundown. However the open probability was reduced following rundown as determined by the onset of MK-801 block of steady-state NMDA currents.

7. Our results suggest that an increase in intracellular calcium leads to channel rundown during whole-cell recording by reducing the open probability of the NMDA channel. Although high concentrations of ATP prevented rundown, we suggest that this action is not a direct result of receptor phosphorylation.

INTRODUCTION

The application of whole-cell recordings to isolated or cultured neurons provides a powerful means to study the function and modulation of voltage- and ligand-gated channels. The extracellular environment is well controlled and solution exchange between the recording pipette and the cytosol allows the introduction of electrolytes and enzymes. Whole-cell dialysis also results in loss of activity ('rundown') of some ion channels, presumably due to washout or alteration of endogenous regulatory molecules (Byerly & Hagiwara, 1982; Fenwick, Marty & Neher, 1982). However, rundown can be used as a bioassay to examine the biochemical basis of channel regulation.

Among the components most sensitive to whole-cell dialysis are intracellular calcium and high-energy phosphates, both of which affect the activity of voltage- and ligand-gated channels (Byerly & Moody, 1984; Chad & Eckert, 1986; Korn & Horn, 1989; Huganir & Greengard, 1990). For example, the activity of $GABA_A$ channels is dependent on ATP (Chen, Steltzer, Kay & Wong, 1990), and $[Ca^{2+}]_i$ (Behrends, Maruyama, Tokutomi & Akaike, 1988; Kano, Rexhausen, Dreessen & Konnerth, 1992). Likewise, the rundown of voltage-gated calcium channels involves Ca^{2+}-dependent proteases and phosphatases (e.g. Chad & Eckert 1986).

Glutamate-activated ion channels also undergo washout or rundown that can be prevented by intracellular dialysis with ATP (MacDonald, Mody & Salter, 1989; Wang, Salter & MacDonald, 1991), an action attributed to phosphorylation. The activity of AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-proprionate) receptors is dependent on cAMP-dependent protein kinase (Greengard, Jan, Nairn & Stevens, 1991; Wang *et al.* 1991). Evidence that receptor dephosphorylation underlies rundown of NMDA channels is less direct, although protein kinase C (PKC) has been reported to enhance whole-cell responses evoked by NMDA (Chen & Huang, 1991; but see Markham & Segal, 1992). NMDA channel rundown and desensitization have been reported to be insensitive to $[Ca^{2+}]_i$ (MacDonald *et al.* 1989; Clark, Clifford & Zorumski, 1990). However, we have recently found that $[Ca^{2+}]_i$ can partially inactivate the NMDA channel (Legendre, Rosenmund & Westbrook, 1993), suggesting the possibility that a Ca^{2+}-dependent process also contributes to channel rundown.

We therefore investigated the role of Ca^{2+} and ATP on currents evoked by NMDA during prolonged whole-cell recordings on cultured hippocampal neurons. Our results suggest that rundown requires an increase in $[Ca^{2+}]_i$. Conditions promoting dephosphorylation of the NMDA receptor did not produce rundown, suggesting that ATP maintains receptor activity by processes other than direct receptor phos-

phorylation. Some of these results have been reported in abstract form (Rosenmund, Legendre & Westbrook, 1991).

METHODS

Cell culture

Cell cultures were prepared as described previously (Legendre & Westbrook, 1990). Briefly, cultured neurons were prepared from the hippocampi of neonatal rats (Sprague–Dawley). Rat pups were anaesthetized with halothane and killed by decapitation. Glass coverslips were coated with collagen (Vitrogen, Collagen Corp., USA) and poly-L-lysine (M_r 30–70, 10^{-5} M in 0·15 M borate, pH 8·4, Sigma Chemical Co., USA). Confluent astrocyte feeder layers were prepared by adding hippocampal cells ($2·5 \times 10^4$ cells per cm^2) 10 days before plating of neurons. For preparation of dissociated neurons, hippocampi were dissected free of meninges, cut into small pieces and slowly agitated for 1 h in low Ca^{2+} medium containing papain (5–20 U/ml, Worthington, USA). The tissue was then triturated using fire-polished pipettes and plated at a density of $5–20 \times 10^3$ per cm^2. Growth medium contained 5% horse serum, 95% minimal essential medium (MEM, Gibco, USA) and a growth supplement including insulin, transferrin, selenium, triiodothyronine, progesterone and corticosterone. Half-changes of medium were made twice weekly.

Recording conditions

Experiments were performed in whole-cell voltage-clamp mode on hippocampal neurons after 5–14 days in culture. Currents were recorded using an Axopatch 1C amplifier (Axon Instruments, USA). Patch pipettes were fabricated from borosilicate glass (TWF 150, WPI, USA) pulled with a conventional two-step puller (Sutter Instruments, USA). Pipettes had 'bubble numbers' ranging from 7·2 to 8·0. After fire-polishing, the inner pipette tip diameter was 1·5–2·5 μM and the resistance was 1–3 MΩ. Pipette solutions included (mM): $CsMeSO_3$, 165; Hepes, 10; Cs_4-BAPTA, 2·4; $CaCl_2$, 0·4; $MgCl_2$, 2. The pH was 7·3 and the osmolarity 310 mosmol/l. The ATP regenerating solution (Forscher & Oxford, 1985; MacDonald *et al.* 1989) included (mM): K_2-ATP, 4; K_2-creatine phosphate, 20; phosphocreatine kinase, 50 U/ml and $MgCl_2$, 6; $CsMeSO_3$ was reduced to 120 mM. In some experiments, caesium gluconate was substituted for $CsMeSO_3$. In most experiments, the protease inhibitor leupeptin (100 μM) was added to the patch pipette. A side-well containing the patch solution was connected via an agar bridge to the bath to offset junction potentials. The series resistance was 60–90% compensated; only recordings with access resistance below 8 MΩ were included in the analysis. Cell capacitance (5–25 pF) was compensated. To ensure stable cell access, the capacitance was monitored with a 7·5 ms hyperpolarizing voltage pulse (−10 mV) preceding each agonist application. Data were acquired using a split-time clock with an acquisition rate of 50 kHz during the voltage pulse and 100 Hz during the agonist application. Currents were low pass filtered at 10 kHz with an 8-pole Bessel filter.

Intracellular dialysis and perfusion

Reagents were introduced into the cell by diffusion from the pipette. Pipettes were backfilled when reagents of high molecular weight were used, and the time between formation of a gigaohm seal and membrane rupture was delayed for 3 min to reduce the exchange time during whole-cell recording. To change pipette solutions, the pipette was perfused as described by Tang, Wang, Quandt & Eisenberg (1990). Briefly, a quartz capillary (30–40 μm i.d.) was inserted within 200–300 μm of the pipette tip, and connected to a reservoir outside the electrode holder via polyethylene tubing (PE-10). The PE tubing was moved between reservoirs to change solutions; solution flow was driven by a negative pressure (−4 to −10 mmHg) through the suction outlet controlled by a pressure transducer (Biotek, USA). Substitution of K^+ with the impermeant cation *N*-methylglucamine (M_r 195) eliminated voltage-dependent K^+ currents within a exchange time constant of 30 s (complete exchange in 90 s); reperfusion restored K^+ currents with a similar time course. As the diffusion from pipette into the cells is approximately proportional to $(M_r)^{1/3}$, this suggests that the 2–3 min of whole-cell recording before the start of agonist application was sufficient to allow near complete exchange of reagents with $M_r < 1000$, assuming no binding within

the cell (Pusch & Neher, 1988), whereas a reagent with a $M_r = 100$ would have an approximate exchange time constant of 5 min. Most intracellular reagents used were in this range except for several enzymes such as alkaline phosphatase (M_r 80).

Drug delivery

The extracellular medium contained (mM): NaCl, 167; KCl, 2·4; Hepes, 10; glucose, 10; $CaCl_2$, 1 and glycine 0·01. The agonist solution contained 10 μM NMDA and either 2 mM or no added Ca^{2+}. The osmolarity was 325 mosmol/l and the pH 7·2. 500 nM TTX, 100 μM picrotoxin and 2 μM strychnine were added to inhibit spontaneous activity as well as ligand-gated chloride channels. Control and agonist solution were applied using an array of flow pipes (400 μm i.d.) positioned within 100–200 μm of the neuron and connected to gravity fed reservoirs. The flow pipe was fabricated using coated quartz glass (Polymicro Tech., USA) that reduced breakage. The tips of the flow pipes were smoothed to allow placement closer to the cells. Each flow pipe was controlled by solenoid valves and was moved with a piezoelectric bimorph (Vernitron, USA). This method led to very consistent agonist delivery with solution exchange times of $18·6 \pm 2·5$ ms ($n = 4$).

Experimental protocols and data analysis

Cells with leak currents > -80 pA at holding potential -60 mV were excluded to avoid non-specific Ca^{2+} entry. To insure adequate voltage-clamp and dialysis, cells with NMDA currents $> 2·5$ nA and large cells with capacitance > 25 pF were excluded. For analysis of rundown 10 μM NMDA was applied every 30 s for 3 s. Membrane currents were recorded using PCLAMP (V.5.5, Axon Instruments, USA) on an IBM-compatible computer and analysed on a MacIntosh using AXOGRAPH software (Axon Instruments, USA). The peak amplitude of agonist-evoked currents was measured by averaging a 100 ms data segment. Rundown was plotted by normalizing the current evoked after 25 min to the current evoked 2 min after the beginning of whole-cell recording. Data is expressed as percentage of control ± S.E.M. Significance was tested using one-way analysis of variance with the Bonferroni–Dunn procedure for multiple comparisons. P levels are given for significance levels $\leqslant 5\%$.

Fluctuation analysis of steady-state NMDA currents was performed as previously described (Mayer, Westbrook & Vyklicky, 1988). Currents were evoked by 2·5 s applications of 10 μM NMDA every 15 s in 0·2 μM Ca^{2+} A 2 s epoch of steady-state current was digitized at 1 kHz. The records were filtered at 500 Hz (8-pole Butterworth). Power spectra were calculated from the ensemble of 30–60 epochs and fitted with the sum of two Lorentzian functions of the form, $S(f) = S(0)/(1 + (f/f_{c1})^2 + S(0)/(1 + (f/f_{c2})^2$ where $S(f)$ is the spectral density, $S(0)$ is the spectral density at 0 Hz, f is the frequency and f_{c1}/f_{c2} are the frequencies at half-power.

Materials

Reagents grade chemicals were obtained from the following sources (parentheses indicate solvent used for stock solution, if other than H_2O): K_2-ATP, potassium creatine phosphate, okadaic acid (dimethylformamide), LR-microcystin (Calbiochem, USA); phosphocreatine kinase/rabbit muscle, calmidazolium (dimethyl sulphoxide, DMSO), staurosporine (DMSO), sodium orthovanadate, phorbol ester dibutyrate (PDBU; ethanol), alkaline phosphatase/E. coli type III-S, TTX, leupeptin, calmodulin/bovine brain, calpain inhibitor 1, ouabain, picrotoxin, bepridil, ATP-γ-S, NMDA, and kainic acid (Sigma Chemical Co., USA) Cs_4-BAPTA, was obtained from Molecular Probes, USA. MK-801 (ethanol) was a gift from Dr Paul Anderson at Merck Sharp and Dohme Research Laboratories, USA.

Phosphatase 1, calcineurin (CaN) inhibitory peptide and calmodulin-dependent protein kinase II (CaM-KII) inhibitory peptide were kindly provided by Dr T. Soderling (Vollum Institute, Portland, OR, USA); the catalytic subunit of cAMP-dependent protein kinase was provided by Dr J. Scott (Vollum Institute). The activity of the reagents in the phosphatase experiments (alkaline phosphatase, phosphatase 1, CaN, CaM, calmidazolium, okadaic acid, microcystin) were verified using an *in vitro* assay (Hashimoto, Perrino & Soderling, 1990) with a phosphopeptide. The activity of staurosporine, PDBU and CaM-inhibitory peptide 281-302 286A was tested on a standard PKC-dependent phosphorylation assay (Kishimoto, Takai, Mori, Kikkawa & Nishizuka, 1980).

RESULTS

Rundown of NMDA currents was prevented by an ATP regenerating solution

Inward currents evoked by 10 μM NMDA are shown in Fig. 1*A*, in the absence (*a*) and presence (*b*) of ATP regenerating solution. Responses were evoked by 3 s

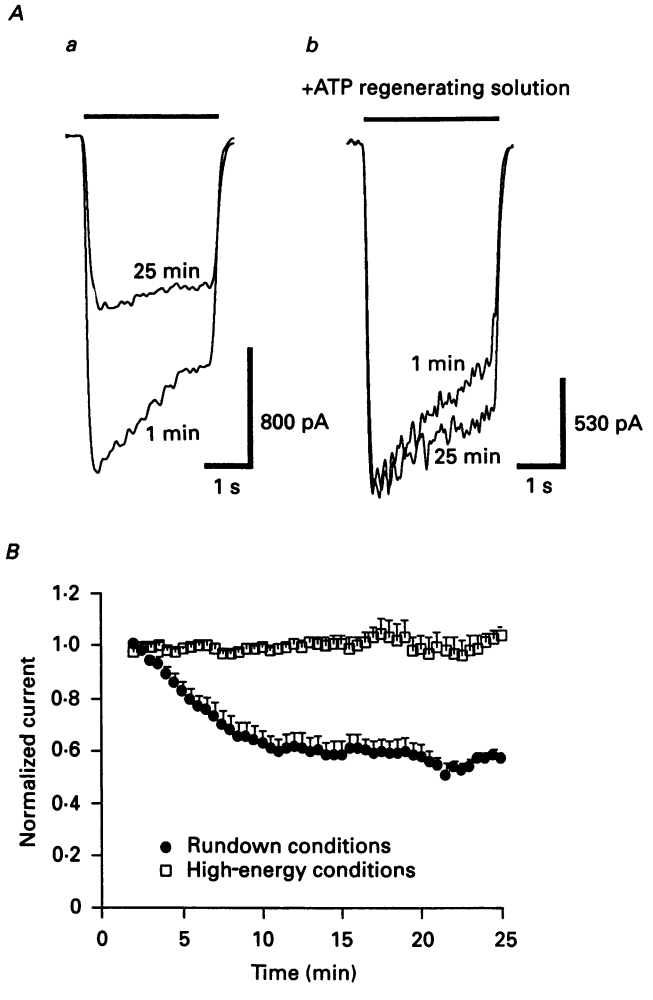

Fig. 1. Rundown of NMDA currents during prolonged whole-cell recording was prevented by ATP regenerating solution. Inward currents were evoked by 3 s applications of 10 μM NMDA at 30 s intervals. *A*, currents evoked after 1 and 25 min of whole-cell recording are superimposed in the absence (*a*) and presence (*b*) of ATP regenerating solution in the pipette. Currents were filtered at 20 Hz for display. *B*, the graph shows the peak current without ATP regenerating solution ('rundown conditions') and with ATP regenerating solution ('high-energy conditions'). Currents were normalized to the first application. Holding potential was −60 mV and rate of agonist application was 0·033 Hz for this and subsequent figures unless otherwise indicated. $[Ca^{2+}]_o$ was 2 mM and patch pipette contained 2·4 mM BAPTA and 0·4 mM Ca^{2+} (20 nM free Ca^{2+}) unless otherwise indicated.

applications of NMDA applied at 30 s intervals. In the absence of ATP regenerating solution, the peak amplitude of the response gradually declined to a steady-state level of 56·2 ± 1·6% after 25 min of recording ($n = 9$). The rundown of the peak current was accompanied by a loss of the slow relaxation ('inactivation') during the

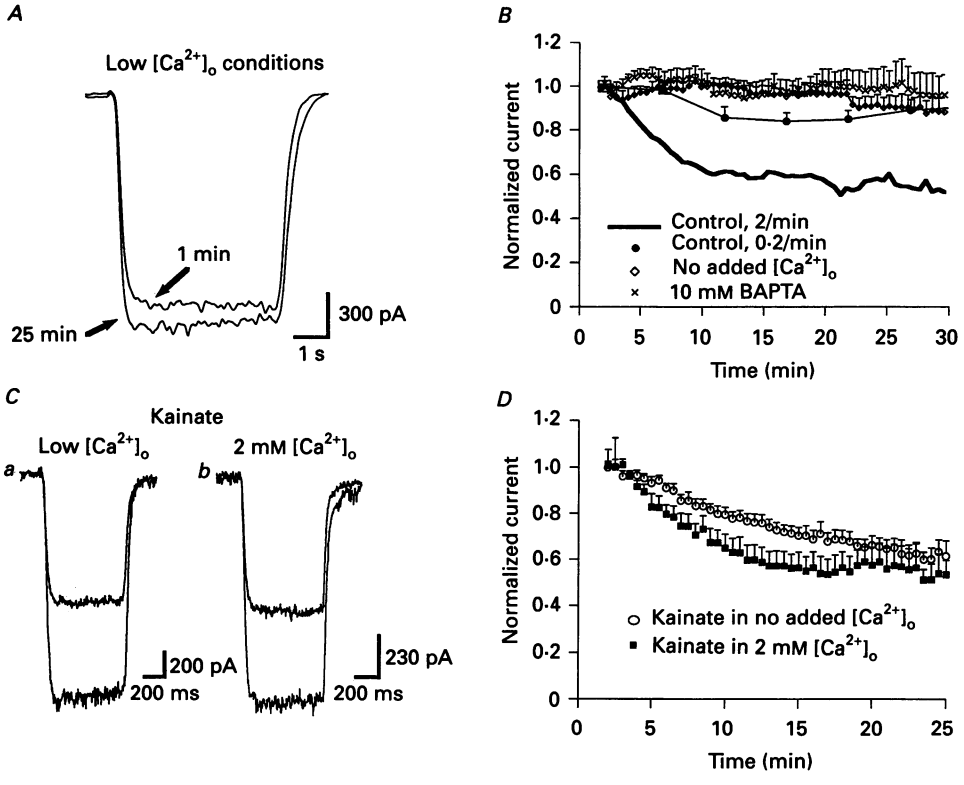

Fig. 2. Rundown of NMDA channels was Ca^{2+}-dependent. *A*, inward currents evoked by NMDA (10 μM) in $[Ca^{2+}]_o$-free solution are superimposed for one neuron at 1 and 25 min of whole-cell recording in the absence of added ATP regenerating solution ('low $[Ca^{2+}]_o$ conditions'). *B*, NMDA currents, evoked in the absence of ATP regenerating solution, are shown in Ca^{2+}-free solution; with BAPTA (10 mM) in the pipette and for low-frequency application rates (0·0033 Hz). Currents under rundown conditions (lower trace) are reproduced from Fig. 1 for comparison. *C* and *D*, rundown of kainate currents was not dependent on $[Ca^{2+}]_o$. Currents evoked by kainic acid (20 μM) at 1 min and 25 min in either low $[Ca^{2+}]_o$ (*Ca*) or 2 mM $[Ca^{2+}]_o$ (*Cb*) are shown. Normalized kainate responses ($n = 7$) are shown in *D*.

3 s NMDA application (Fig. 1*Aa*). We have previously demonstrated that inactivation is due to increases in intracellular calcium (Legendre *et al.* 1993). In the presence of ATP regenerating solution, NMDA currents were stable for up to 40 min (103·2 ± 1·6%, $t = 25$ min; $n = 10$, $P < 0·0001$). However, Ca^{2+}-dependent inactivation was not prevented by the ATP regenerating solution (Fig. 1*Ab*).

Channel rundown in our experiments was similar in degree to that observed by MacDonald *et al.* (1989). The time course of rundown was relatively slow, reaching half-maximal reduction after approximately 6 min (Fig. 1*B*). The rate of the Ca^{2+}-

dependent inactivation also slowed during the first minutes of whole-cell recording in the presence of ATP regenerating solution (Fig. 1*Ab*). Because the rate of inactivation is dependent on buffering of $[Ca^{2+}]_i$ (Legendre *et al.* 1993), the slowing of inactivation may reflect diffusion of exogenous buffer into the cell. Therefore, we waited 2 min after establishing whole-cell recording before applying NMDA in Ca^{2+}-containing solutions. In some experiments short agonist pulses (300 ms) in Ca^{2+}-free medium were applied before the first agonist application in Ca^{2+}-containing medium. The amplitude of the current remained stable or increased slightly during this initial period ($107{\cdot}6 \pm 5{\cdot}6\%$, $n = 13$).

Rundown was Ca^{2+}-dependent

Ca^{2+}-dependent inactivation of NMDA currents disappeared in parallel with current rundown in the absence of ATP regenerating solution. The maximal current inactivation (57% of control, Legendre *et al.* 1993) is also similar to the extent of current loss during rundown. Thus we tested whether rundown might also be mediated by $[Ca^{2+}]_i$. As expected, inactivation was no longer present in Ca^{2+}-free solutions (Fig. 2*A*). In addition, no significant current rundown occurred in Ca^{2+}-free solutions even in the absence of ATP regenerating solution ($99{\cdot}4 \pm 3{\cdot}1\%$; $n = 9$, $P < 0{\cdot}0001$; Fig. 2*A*). Increases in the length (0·3–5 s) or frequency (0·0033–0·1 Hz) of agonist applications increased rundown in Ca^{2+}-containing solutions, but had no effect in Ca^{2+}-free solutions ($n = 8$), suggesting that rundown did not result from ligand-mediated desensitization. In fact responses remained stable in Ca^{2+}-free solutions as long as the seal resistance was maintained. A slight increase of the peak amplitude accompanied by a fast component of desensitization occasionally occurred after 30 min or more, presumably due to glycine-independent desensitization that is prominent in outside-out patches (Sather, Johnston, Henderson & Ascher, 1990).

If rundown results from increases in $[Ca^{2+}]_i$, decreasing the frequency of NMDA applications or increasing intracellular calcium buffers would be expected to reduce rundown. Consistent with this, when NMDA was applied every 5 min in Ca^{2+}-containing solutions, the rundown was nearly abolished ($90{\cdot}1 \pm 5{\cdot}8\%$, $n = 5$, $P < 0{\cdot}0001$). Rundown was also reduced when BAPTA was increased to 10 mM in the patch pipette ($95{\cdot}8 \pm 3{\cdot}8\%$, $n = 6$, $P < 0{\cdot}0001$). These results are summarized in Fig. 2*B*. The Ca^{2+} dependence of NMDA channel rundown was receptor-specific as rundown of kainate responses was similar in the absence ($61{\cdot}7 \pm 4{\cdot}8\%$) and presence ($52{\cdot}6 \pm 8{\cdot}4\%$) of extracellular Ca^{2+} ($n = 7$, Fig. 2*C* and *D*).

To obtain more direct evidence that rundown was dependent on $[Ca^{2+}]_i$, we perfused the patch pipette using the technique described by Tang *et al.* (1990). In this way, $[Ca^{2+}]_i$ could be altered independently on transmembrane calcium flux through NMDA channels. As shown in Fig. 3*A*, responses evoked by NMDA (10 μM) in Ca^{2+}-free solution were stable during the first 10 min of recording when the control patch solution contained 20 nM free Ca^{2+} (2·4 mM BAPTA/0·4 mM Ca^{2+} with no added ATP regenerating solution). However, when the pipette was perfused with 1 mM free Ca^{2+} (2·4 mM BAPTA/3·4 mM $CaCl_2$), the current declined in 7 min to $67{\cdot}4 \pm 4{\cdot}9\%$ ($n = 4$). The inhibition was reversible on reperfusion with control buffer ($92{\cdot}5 \pm 5{\cdot}2\%$, $n = 4$). To more carefully control the $[Ca^{2+}]$ in the perfusate, we used the low affinity chelator dibromo BAPTA (Adler, Augustine, Duffy & Charlton, 1991). In three neurons,

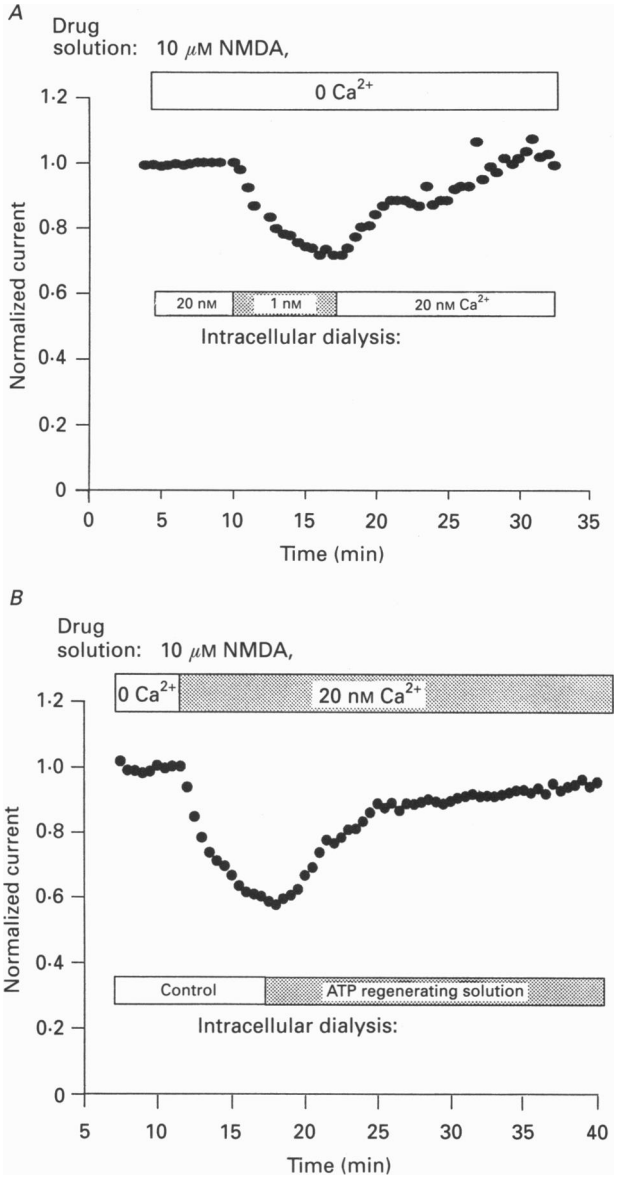

Fig. 3. Intracellular perfusion with high Ca^{2+}-produced reversible inhibition of the NMDA current and ATP counteracted Ca^{2+}-dependent rundown. *A*, currents were evoked under low external calcium conditions. The cell interior was then perfused for 7 min with a solution containing 1 mM free Ca^{2+}. The NMDA current declined to 71 % of control in the absence of $[Ca^{2+}]_o$, then recovered upon reperfusion with control buffer (2·4 mM BAPTA/0·4 mM Ca^{2+}). *B*, in another neuron, raising $[Ca^{2+}]_o$ during regular NMDA applications resulted in rapid rundown that was reversed by intracellular perfusion with ATP regenerating solution. The currents in the presence of $[Ca^{2+}]_o$ were normalized to the last application in Ca^{2+}-free solution to account for the decrease in single channel conductance.

perfusion with 15 μM free Ca^{2+} (10 mM dibromo BAPTA/9 mM $CaCl_2$) gave similar results. The rate and extent of current inhibition was similar to that of NMDA channel rundown in Ca^{2+}-containing solutions (Fig. 3*B*).

ATP might prevent rundown by either facilitating the clearance of calcium (Byerly & Moody, 1984; Miller, 1991) or neutralizing its effect. To examine the

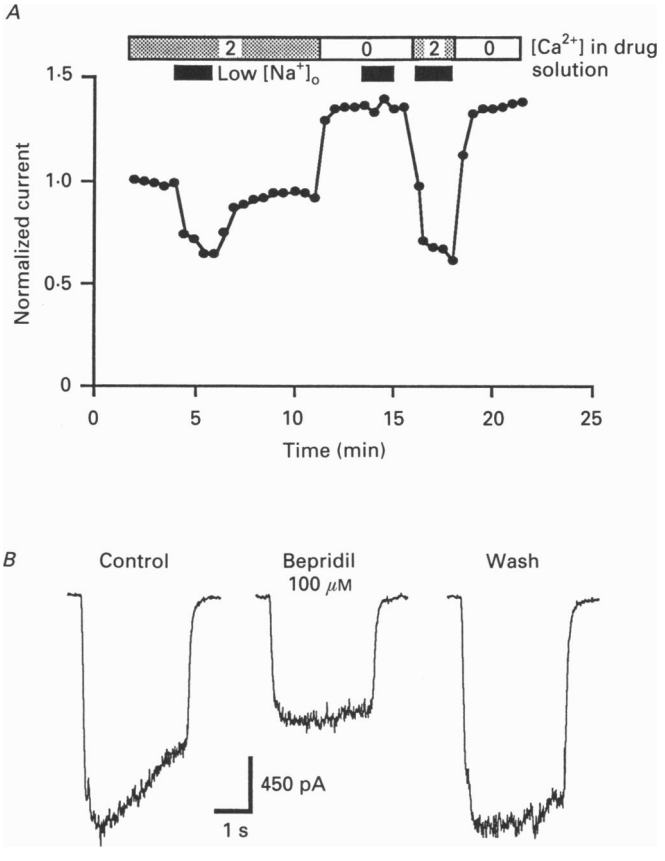

Fig. 4. The Na^+–Ca^{2+} exchanger is required for maintenance of NMDA currents. *A*, NMDA currents were evoked in a neuron under high-energy conditions as outlined in Fig. 1. Reduction in $[Na^+]_o$ to 42 mM during the 27 s intervals between NMDA applications (filled bar) induced channel rundown that was completely reversible when 167 mM $[Na^+]_o$ was reintroduced. Repeat exposure to low $[Na^+]_o$ had no effect if NMDA was applied in Ca^{2+}-free solutions. The increased current in Ca^{2+}-free solutions is due to the larger channel conductance. On return to Ca^{2+}-containing agonist solutions, low $[Na^+]_o$, again reversible, inhibited the current. *B*, block of the Na^+–Ca^{2+} exchanger with 100 μM bepridil also produced a 50% inhibition after five NMDA applications that was fully reversible 8 min after washout of bepridil.

relationship between Ca^{2+} and ATP, we tested whether intracellular perfusion with ATP regenerating solution was sufficient to reverse rundown (Fig. 3*B*). Rundown, induced by switching from Ca^{2+}-free to a Ca^{2+}-containing agonist solution, reached $64{\cdot}5 \pm 3{\cdot}6\%$ ($n = 4$) after 5 min. Subsequently, intracellular perfusion with ATP

regenerating solution resulted in full recovery (94·6 ± 4·7 %, Fig. 3*B*) despite the continued agonist applications. Thus ATP is sufficient to overcome Ca^{2+}-dependent rundown.

The Na^+–Ca^{2+} exchanger contributed to the maintenance of the NMDA response

The above results clearly demonstrate that increases in $[Ca^{2+}]_i$ can mimic channel rundown. In whole-cell recording exogenous buffers clamp the steady-state $[Ca^{2+}]_i$, but are less efficient in clamping Ca^{2+} transients, a function largely controlled by the Na^+–Ca^{2+} exchanger in intact neurons (Blaustein, 1988). If rapid clearance of Ca^{2+} transients is required to prevent channel rundown, block of the exchanger would be expected to induce rundown. We tested this possibility by reducing the sodium gradient during the interval between pulses of NMDA. The agonist solution contained 167 mM $[Na^+]_o$, 2 mM $[Ca^{2+}]_o$ and ATP regenerating solution was included in the pipette (Fig. 4*A*). After a 2 min baseline period, the neurons were bathed in low $[Na^+]_o$ (42 mM) during the 27 s between agonist applications. This sodium concentration is expected to inhibit the exchanger, but should not induce Ca^{2+} influx via the exchanger (Blaustein, 1988). The current declined rapidly to 70·6 ± 5·7 % (n = 8) after 2 min in low $[Na^+]_o$ and completely recovered on return to 167 mM $[Na^+]_o$ (96·3 ± 4·6 %, n = 8). The NMDA current following the first exposure to low $[Na^+]_o$ was unchanged if low $[Na^+]_o$ was introduced late during the 27 s interpulse interval, as is the case for the cell shown in Fig. 4*A*. This suggests that significant Ca^{2+} clearance by the exchanger occurs within 10–15 s following a NMDA application.

To exclude the possibility that reversal of the Na^+–Ca^{2+} exchanger was responsible for the reduction in current, NMDA was subsequently applied in Ca^{2+}-free solutions. There was an initial increase in the current amplitude, due to the increase in single channel conductance in low Ca^{2+} solutions (Jahr & Stevens, 1987; Ascher & Nowak, 1988). However, inhibition of the exchanger with low $[Na^+]_o$ now had no effect on the NMDA current. Thus Ca^{2+} that enters through NMDA channels was solely responsible for the reduced current during inhibition of the Na^+–Ca^{2+} exchanger.

We also inhibited the exchanger by substituting lithium for sodium in both the agonist and control solutions, or with bepridil (Kaczorowski, Slaughter, King & Garcia, 1989). Lithium is permeable through NMDA channels, but does not support the exchanger (Baker & DiPolo, 1984). Lithium substitution produced a similar current inhibition (74·1 ± 3·3 %, n = 6) as in 42 mM $[Na^+]_o$. However, in two cells, the current initially increased before declining to 78 and 69 % of control. We attributed the transient increase to synaptic release of glutamate because spontaneous synaptic activity even in presence of TTX was also observed in these cells. Inhibition of the Na^+–Ca^{2+} exchanger with 100 μM bepridil also produced a reversible decrease in the current (67·7 ± 5·1 %, n = 5, Fig. 4*B*). In two of these cells, a transient increase in the current and in spontaneous synaptic activity developing within 3–10 s, similar to that seen with lithium substitution. These results suggest that the Na^+–Ca^{2+} exchanger plays a crucial role in the clearance of Ca^{2+} transients even during whole-cell dialysis with BAPTA and suggests that Ca^{2+} transients trigger NMDA channel rundown.

Is rundown reversible?

The intracellular perfusion experiments suggest that the action of intracellular calcium is reversible, although MacDonald *et al.* (1989) noted a lack of spontaneous recovery of NMDA current washout in the absence of ATP regenerating solution.

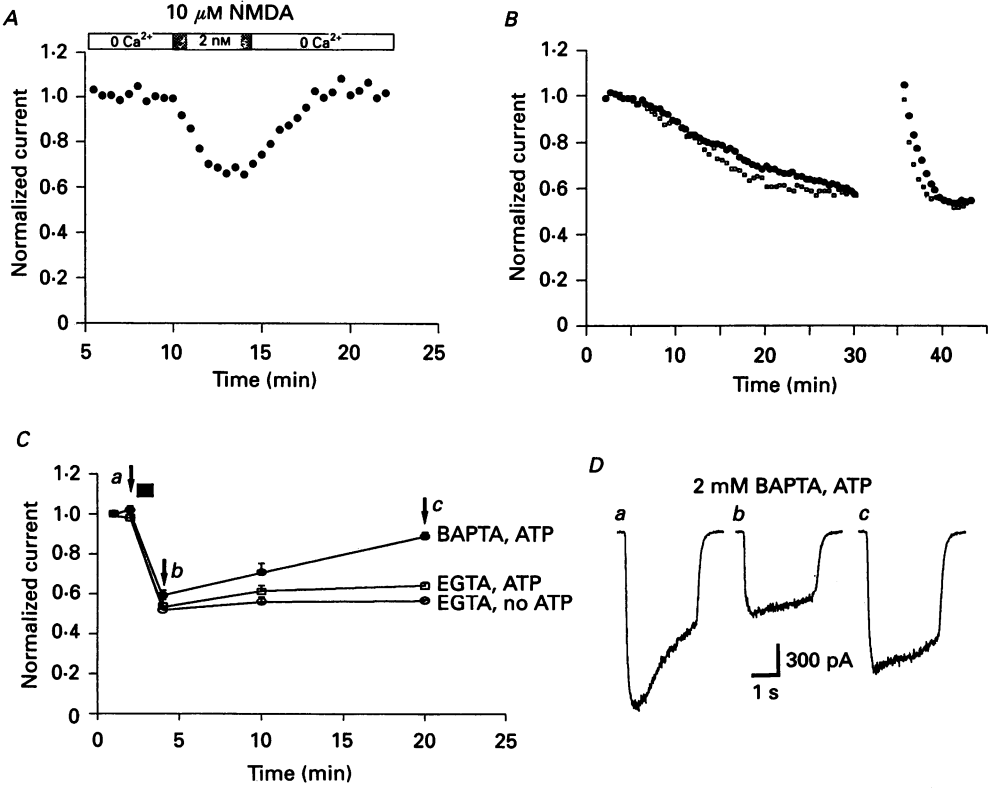

Fig. 5. The reversibility of channel rundown was condition dependent. *A*, in the absence of ATP regenerating solution, currents evoked by NMDA were reversibly inhibited when extracellular [Ca^{2+}] was increased to 2 mM for 5 min. The currents in 2 mM $[Ca^{2+}]_o$ were normalized. *B*, rundown of two neurons dialysed with control buffer and 2 mM Ca^{2+} in the agonist solution are superimposed. After agonist applications were suspended for 5 min, the current was fully restored. Subsequent applications resulted in a much more rapid rundown. *C*, the recovery after large Ca^{2+} transients were examined. Cells were exposed to five to ten 5 s pulses of 1 mM NMDA in 2 mM [Ca^{2+}] for 1–2 min (filled bar). Subsequent NMDA responses after 1, 11 and 21 min recovered for cells dialysed with BAPTA and ATP regenerating solution, but not for cells dialysed with EGTA in the presence or absence of ATP regenerating solution. Included in the patch solution were 100 μM leupeptin and 50 μM calpain inhibitor 1. *D*, responses for one cell dialysed with BAPTA are shown at times indicated by *a*, *b* and *c* in *C*.

Plausible explanations for this apparent discrepancy include the existence of several underlying mechanisms or that channel activity can no longer recover if the cell cannot maintain calcium homeostasis. We conducted several experiments to examine these possibilities. As shown in Fig. 5*A*, rundown in Ca^{2+}-containing solutions was reversible when calcium was removed from the agonist solution (91·4 ± 5·5 %, $n = 5$).

We also tested the reversibility of rundown by interrupting the NMDA applications for 5 min. Figure 5*B* shows superimposed responses of two cells that ran down to 60% of control after 30 min of recording. However, the current recovered completely after a 5 min pause in agonist applications (95·6 ± 4·5%, $n = 6$). This was followed by

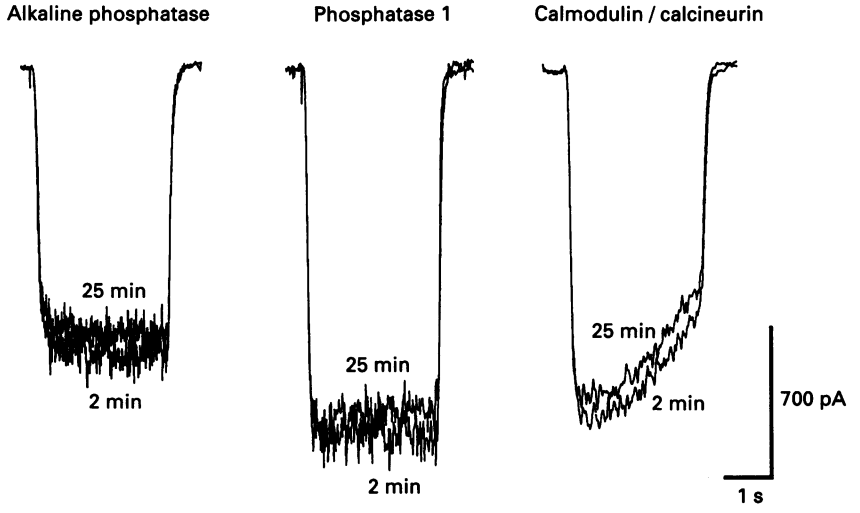

Fig. 6. Intracellular dialysis with phosphatases did not inhibit NMDA currents. Currents evoked by NMDA at 2 and 25 min of whole-cell recording are superimposed for 3 neurons. Alkaline phosphatase (100 μg/ml), phosphatase 1 (250 ng/ml) or calcineurin (120 U/ml) in combination with calmodulin (600 U/ml) had no effect on the peak amplitude of the current. For alkaline phosphatase and phosphatase 1, currents were evoked in Ca^{2+}-free agonist solutions without ATP regenerating solution in the pipette. For calcineurin, the pipette contained ATP regenerating solution, and the agonist solution contained 2 mM Ca^{2+} so that calcium entry could activate the phosphatase.

much more rapid rundown, consistent with a progressive decrease in the ability of the neuron to recover from a series of Ca^{2+} transients.

However, we could evoke irreversible rundown using protocols that favoured the development of large uncontrolled calcium transients. For example, if agonist applications were begun immediately after establishment of whole-cell recording using pipettes buffered with 10 mM EGTA, short pulses of high concentrations of agonist (200 ms, 2/min, 250 μM NMDA) resulted in rapid and irreversible rundown ($n = 5$). Because the slowly acting buffer EGTA is much less effective than BAPTA in controlling submembrane calcium transients (Byerly & Moody, 1984; Adler *et al.* 1991), EGTA may be insufficient to buffer NMDA-evoked Ca^{2+} transients. This would suggest that even under constant buffer conditions, large and uncontrolled Ca^{2+} transients can overcome the neuron's capacity to recover from rundown. To test this directly, we examined recovery from rundown after 1–2 min of intense stimulation (5–10 applications of 1 mM NMDA, duration 5 s each). The protease inhibitors 100 μM leupeptin and 50 μM calpain inhibitor 1 were added to the patch solution. We compared three buffer conditions: 10 mM EGTA with no added Ca^{2+} in the presence or absence of ATP regenerating solution, and 2·4 mM BAPTA with

0·4 mM Ca^{2+} in the presence of ATP regenerating solution. In all three cases, the response to the second application was immediately reduced by 40% and then stabilized at approximately 50% of control (Fig. 5*C*). Rundown was much more rapid than in our standard protocol (see Fig. 1). For EGTA in the absence of ATP regenerating solution, rundown was irreversible (57·0 ± 0·02%, $n = 4$ at $t = 20$ min, Fig. 5*C*). The addition of ATP regenerating solution only marginally increased the recovery (64·3 ± 1·8%, $n = 4$ at $t = 20$ min). However in the presence of BAPTA and ATP regenerating solution, the response showed a slow recovery to 89·1 ± 2·1%, ($n = 4$, Fig. 5*C* and *D*). This suggests that rundown becomes irreversible when the Ca^{2+} transient exceeds a certain threshold.

Dephosphorylation of the NMDA channel did not appear to be responsible for rundown

The above results suggest that rundown of NMDA currents in whole-cell recordings depend primarily on the size and frequency of evoked Ca^{2+} transients, and that ATP counteracts the effect of Ca^{2+} directly or indirectly. We employed three basic protocols to examine whether the action of Ca^{2+} and ATP on rundown involved receptor phosphorylation. These were *rundown* (2 mM $[Ca^{2+}]_o$, no ATP regenerating solution); *high energy* (2 mM $[Ca^{2+}]_o$, ATP regenerating solution) and *low external calcium* (no added $[Ca^{2+}]_o$, no ATP regenerating solution). We first tested whether dialysis with phosphatases could mimic rundown under low external calcium conditions (Fig. 6). Neither alkaline phosphatase (100 μg/ml; 97·1 ± 3·6%; $n = 6$) nor phosphatase 1 (250 ng/ml; 99·7 ± 4·6%; $n = 7$) had any effect on the amplitude of the current during 25 min of whole-cell recording. To test the effect of the Ca^{2+}-dependent phosphatase, calcineurin (120 U/ml) and calmodulin (600 U/ml) were added to the pipette under high-energy conditions; this was also ineffective in promoting rundown (99·3 ± 2·9%, $n = 5$). These results suggest that NMDA channels can function in the dephosphorylated state. However, in the presence of transmembrane calcium influx and ATP regenerating solution, we cannot exclude the possibility that phosphorylation of regulatory elements (e.g. the Na^+–Ca^{2+} exchanger, Miller, 1991) influences channel rundown. For example, alkaline phosphatase had a small effect in the high-energy conditions, but this did reach statistical significance (100 μg/ml; 89·8 ± 5·9%; $n = 6$).

ATP could prevent rundown by mechanisms other than phosphorylation. For example, the non-specific ATPase inhibitor vanadate (10 μM) rapidly inhibited NMDA currents under high-energy conditions (77·0 ± 7·5%, $n = 7$, $P < 0·0001$). Because ATP analogues differ in their efficacy as substrate for kinases or ATPases, we compared the effect of inorganic phosphate and ATP analogues on rundown. Inorganic phosphate (25 or 125 mM) did not prevent rundown (60·2 ± 3·8%; $n = 7$ and 62·9 ± 4·7%, $n = 5$), suggesting that ATP is required to prevent rundown (Fig. 7*A*). However, rundown was significantly delayed by phosphate. After 8 min of whole-cell recording, the response was 89·6 ± 3·1% ($P < 0·001$) for 25 mM phosphate and 88·5 ± 6·3% ($P < 0·001$) for 125 mM phosphate compared to 68·2 ± 5·1% in the absence of phosphate or ATP regenerating solution (Fig. 7*B*). Thus inorganic phosphates may act as calcium buffers or alter the endogenous ATP:ADP ratio. Submillimolar concentrations of ATP should be sufficient to fully activate kinases;

however, 4 mM ATP in the absence of the regenerating system was not sufficient to completely prevent rundown (76·6±7·3%; $n = 6$, $P < 0·0001$). Likewise, substitution of ATP-γ-S, although less effective than ATP as a kinase substrate, would be expected to shift the equilibrium toward the phosphorylated state as

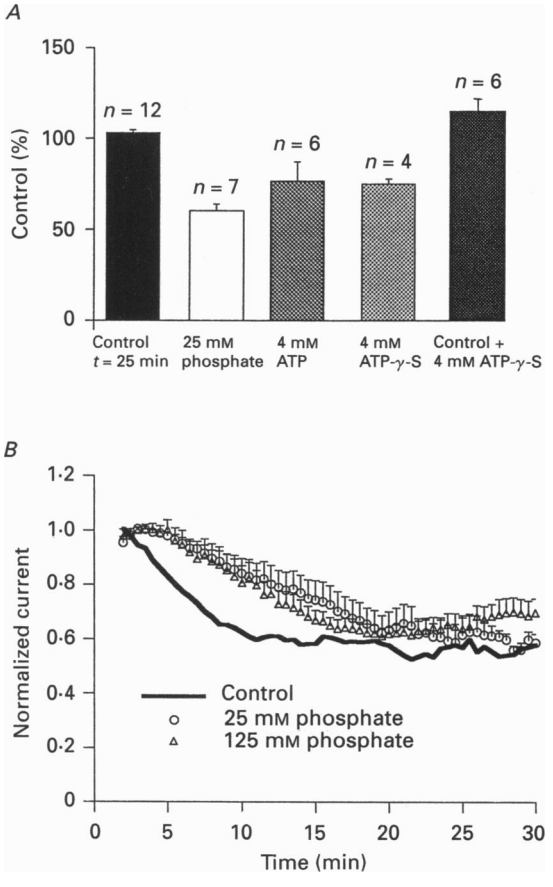

Fig. 7. The efficacy of different phosphates in preventing rundown. *A*, cells were dialysed for 25 min with different phosphates. NMDA currents (means±S.E.M.) at 25 min were normalized to the peak current after 2 min of recording. Rundown was not prevented with 25 mM phosphate. 4 mM ATP as well as 4 mM ATP-γ-S were significantly less effective than the ATP regenerating solution (control). However, 4 mM ATP-γ-S added to the ATP regenerating solution increased the response slightly above control. *B*, the time course of rundown was delayed for cells dialysed with 25 or 125 mM phosphate. Rundown in the absence of phosphates (control) is reproduced from Fig. 1 for comparison (lower trace).

thiophosphates are resistant to dephosphorylation. However, 4 mM ATP-γ-S in the regenerating solution was much less effective (75·0±2·9%; $n = 4$, $P < 0·0001$) than ATP regenerating solution. Addition of 4 mM ATP-γ-S to the 4 mM ATP in the regenerating solution increased the response above the control levels (120·5±7·1%; $P = 0·0037$), $n = 6$), suggesting that 4 mM ATP is a submaximal concentration in preventing rundown.

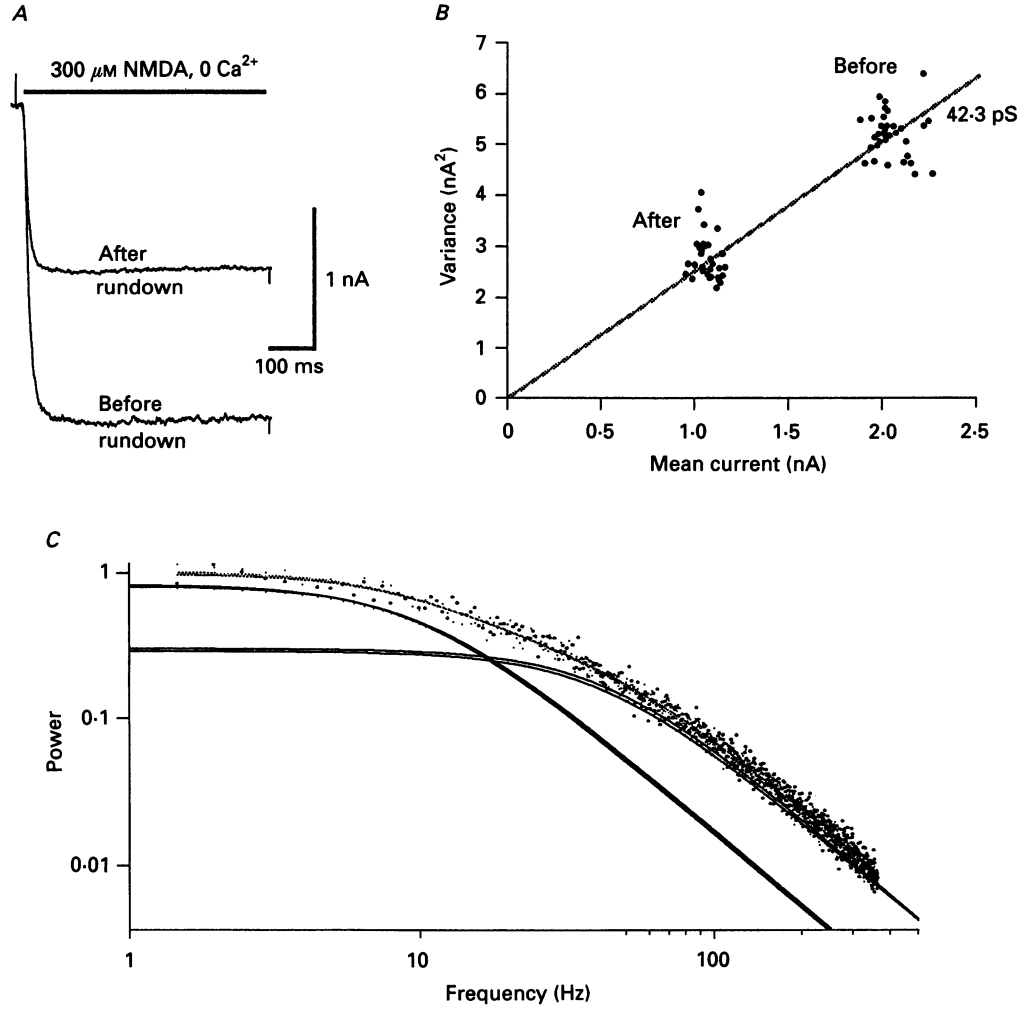

Fig. 8. Rundown did not result from a decrease in agonist affinity or channel lifetime. *A*, the current evoked by high concentrations of NMDA (300 μM) in [Ca^{2+}]-free solution ran down to the same degree as currents evoked by low concentrations of agonist. *B*, fluctuation analysis of steady-state ensemble currents evoked by 2·5 s 10 μM NMDA pulses in 0·2 mM $[Ca^{2+}]_o$ was used to calculate the mean–variance relationship before and after rundown. The line represents the calculated conductance before rundown ('before'). The conductance was 43·0 pS after rundown for this neuron ('after'). *C*, the normalized power spectra for the data shown in *B*. The spectra were best fitted with the sum of two Lorentzians. Before rundown (large dots) the corner frequencies for this neuron were 41·2 and 9·2 Hz corresponding to mean open times of 6·5 and 1·5 ms. After rundown (small dots), the shoulder frequencies were 39·4 and 9·4 Hz corresponding to mean open times of 6·2 and 1·5 ms, respectively. The holding potential was −60 mV.

To evaluate further the role of phosphorylation on channel rundown, we tested a series of kinase and phosphatase activators and inhibitors. Reagents were added to the patch solution under high-energy conditions to evaluate their ability to modulate rundown, or under rundown conditions to see if a reagent could prevent rundown.

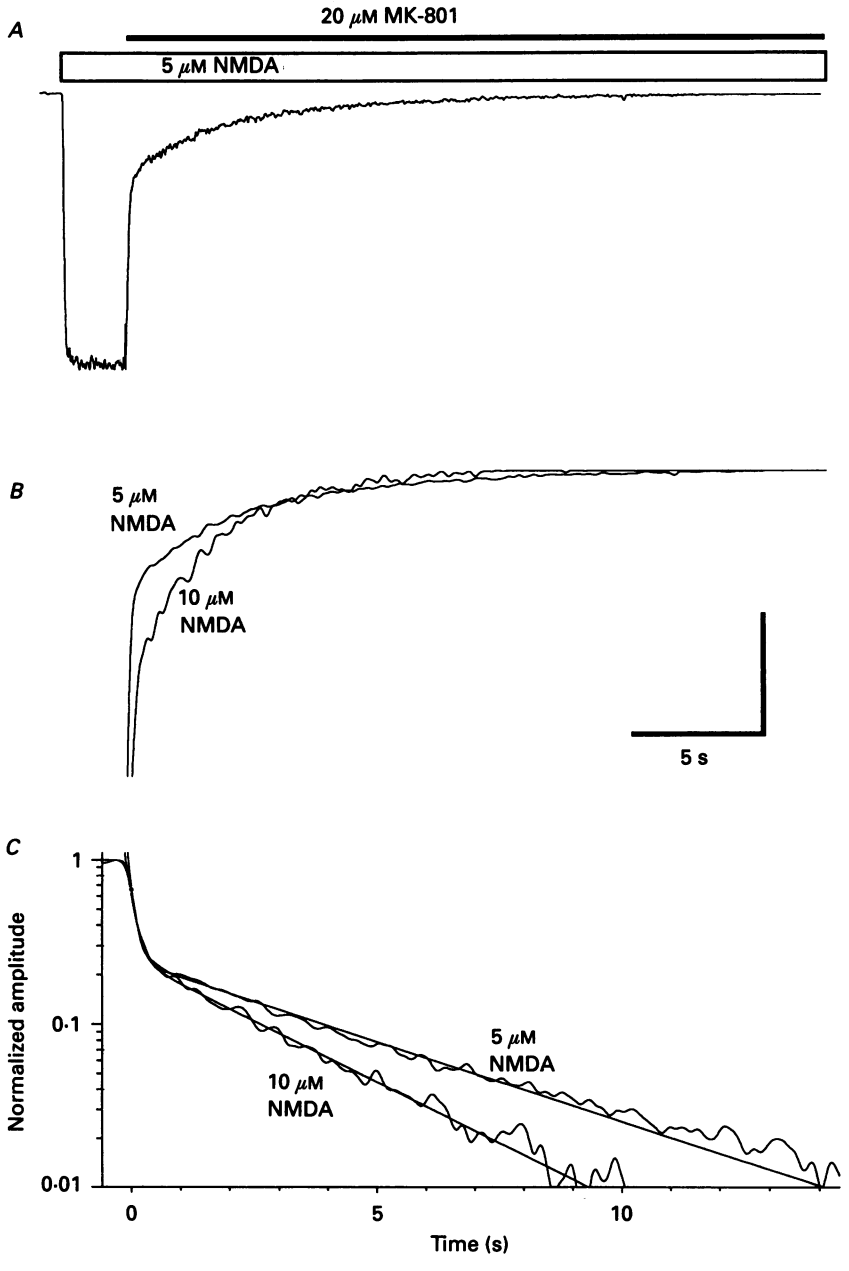

Fig. 9. Changes in open probability were measured by the time course of channel block by MK-801. *A*, current evoked by a 2 s application of 5 μM NMDA decayed with fast and slow components when exposed to 5 μM NMDA + 20 μM MK-801. The decay was fitted with the sum of two exponentials with time constants of 106 ms (coefficient 70·6%) and 4·38 s (coefficient 29·3%). The holding potential was −60 mV. *B*, the decay for the cell shown in *A* is compared to that of a second neuron in a higher concentration of 10 μM NMDA. The two neurons had comparable steady-state currents evoked by 10 μM NMDA. The slow component of the decay was prolonged for the 5 μM NMDA response, but the charge transfer in the presence of MK-801 was equal (see text for details). The slow decay in 10 μM NMDA had a time constant of 2·8 s (coefficient 31·1%). Calibration bar is 170 pA

The activity of the reagents was tested in biochemical assays, concentrations used in the pipette were supramaximal and 2–3 min were allowed before the start of recording to facilitate adequate intracellular dialysis (see Methods). The kinase inhibitor staurosporine (1 μM) resulted in partial rundown (79·8 % ± 6·4; $n = 6$, $P < 0{\cdot}0001$) after 25 min under high-energy conditions, but this could be overcome by a slight reduction in the application rate (2/min to 1·3/min; 94·2 ± 4·4 %, $n = 3$). The CaM-KII inhibitory peptide 281-302/A286 (50 μM, Soderling, 1990) in a concentration that fully blocks CaM-KII had no significant effect (93·8 ± 3·6 %, $n = 7$). The catalytic subunit of protein kinase A (9 μg/ml, $n = 4$) or 100 nM phorbol-D-butyrate also did not increase the response above control levels. The current amplitude at 25 min was 96·8 ± 5·1 % ($n = 4$) and 104·6 ± 3·0 % ($n = 12$), respectively. Likewise, phosphatase inhibitors had no significant effect on rundown. Under rundown conditions, the current at 25 min was 65·6 % ± 5·1, ($n = 5$) in the presence of 5 μM okadaic acid, 60·5 ± 3·3 % ($n = 5$) in the presence of 10 μM LR-microcystin and 61·1 ± 3·0 ($n = 5$) in the presence of 100 μM CaN inhibitory peptide A457-491 (Hashimoto *et al.* 1990).

These results suggest that the Ca^{2+} dependence of rundown involve processes other than phosphorylation–dephosphorylation. However rundown was Ca^{2+}-selective and calmodulin-dependent. Rundown did not occur when 2 mM Ba^{2+} was substituted for Ca^{2+} (89·4 ± 2·1 %, $n = 12$, $P < 0{\cdot}0001$) and the calmodulin inhibitor calmidazolium (CMZ, 10 μM) significantly retarded rundown (83·6 ± 4·5 %, $n = 11$, $P < 0{\cdot}0001$). CMZ also appeared to increase slightly the response in the presence of ATP regenerating solution (110·1 ± 4·2 %; $n = 9$), although this did not reach statistical significance.

A reduction in P_o underlies rundown

Ca^{2+}-dependent rundown is not apparent in outside-out membrane patches (Sather *et al.* 1990; Rosenmund & Westbrook, unpublished). Therefore we used several whole-cell methods to examine the changes in the single channel properties responsible for rundown. The whole-cell current (I_{wc}) can be represented by,

$$I_{wc} = ni(\mathrm{MOT})P_o,$$

where n is the number of available channels, i is the single channel current, MOT is the mean open time, and P_o is the open probability. P_o is dependent on agonist affinity and gating into the open state. Rundown did not result from a decrease in agonist affinity as similar degrees of rundown (52·0 ± 3·4 %, $n = 6$) were observed at supramaximal concentrations of NMDA (300 μM, Fig. 8*A*).

To estimate the channel conductance and MOT before and after rundown, fluctuation analysis was performed on steady-state currents evoked by 2·5 s applications of 10 μM NMDA in 0·2 mM $[Ca^{2+}]_o$. For each neuron, 30–60 epochs (2 s duration) were collected and the variance of each epoch was plotted *versus* the mean

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for the 5 μM NMDA response and 180 pA for the 10 μM response. *C*, semilogarithmic plot of the current decays in MK-801 with superimposed exponential curve fits are shown for the neurons in *A* and *B*.

current (Fig. 8*B*). The unitary conductance was calculated from the equation $\gamma = \sigma/I(V_h - V_r)$, where σ is the variance, I is the mean whole-cell current and $(V_h - V_r)$ is the driving force. For five neurons the conductance before rundown was $42{\cdot}8 \pm 2{\cdot}9$ pS, consistent with prior estimates of γ by fluctuation analysis (Mayer *et al.* 1988). Following rundown there was no significant change in the conductance ($43{\cdot}7 \pm 2{\cdot}8$ pS). The MOT was estimated by fitting the power spectra with the sum of two Lorentzians (Fig. 8*C*). Before rundown the corner frequencies (f_{c1} and f_{c2}) were $40{\cdot}5 \pm 2{\cdot}2$ and $9{\cdot}2 \pm 0{\cdot}9$ Hz, corresponding to time constants of 4·0 and 17·3 ms. Following rundown, there was no significant change in MOT. The corner frequencies were $40{\cdot}1 \pm 1{\cdot}1$ and $10{\cdot}0 \pm 0{\cdot}7$ Hz with time constants of 4·0 and 15·9 ms.

By exclusion, the results of fluctuation analysis suggest that rundown is due to either a change in the number of channels or in P_o. To estimate these parameters, we analyse the open channel block of whole-cell currents by MK-801. The irreversible block by MK-801 ($\tau_{recovery} \approx 90$ min, Huettner & Bean, 1988) has been used to estimate the P_o of NMDA channels in outside-out patches (Jahr, 1992). If MK-801 is rapidly applied during a steady-state NMDA whole-cell current, the open time of each open channel will be reduced as a function of [MK-801]. The current will then decay as channels open and become irreversibly blocked. As a result, the decay is dependent on the sum of rates leading into the open state and a reduction in P_o will result in slowing of this decay. Likewise, the charge transfer during the decay provides an estimate of the number of active channels.

The experimental protocol is shown in Fig. 9*A*. After the current evoked by NMDA in a low extracellular calcium (0·2 mM) reached steady state 20 μM MK-801 was added in the continuous presence of agonist. We tested the validity of our approach by comparing the blocking rates of MK-801 in 5 and 10 μM NMDA. The current amplitude in 5 μM NMDA was approximately 50% of the response at 10 μM NMDA (Fig. 10*C*), and thus the estimated P_o should be reduced by 50% in the lower concentration of agonist. The current decayed to $< 1\%$ of the initial value and was irreversible as a subsequent application of NMDA 1 min later showed no recovery (data not shown). The decay could be fitted with two exponentials. The initial decay had a time constant of $114{\cdot}1 \pm 10{\cdot}4$ ms and a coefficient of $68{\cdot}6 \pm 0{\cdot}6\%$ ($n = 17$), presumably reflecting the equilibration of MK-801 at the cell. The percentage of block was independent of the concentration of NMDA and is in agreement with that predicted for a MK-801 binding rate of 23·7–25 $\mu M^{-1}\ s^{-1}$ (Huettner & Bean, 1988; Jahr, 1992) and a channel closing rate of 200 s^{-1} (e.g. Clements & Westbrook, 1991). However the slow decay was prolonged as the agonist concentration was decreased, i.e. as P_o decreased. The decay in 5 and 10 μM NMDA for two cells are shown in Fig. 9*B*. As expected, the decay was slower in 5 μM NMDA, but the number of channels are measured by the charge transfer (i.e. the area under the curve during the decay) was unchanged (Fig. 9*B* and 10*Cb*).

Following rundown, the decay in MK-801 was also markedly slowed, consistent with a reduction in P_o (Fig. 10*A* and *B*). However there was no significant change in the charge transfer (Fig. 10*Cb*), suggesting that the number of channels remained constant. The currents in Fig. 10*A* were normalized to the steady-state current amplitude immediately preceding the application of MK-801. The estimates of P_o and charge transfer for a decrease in agonist concentration and for rundown are

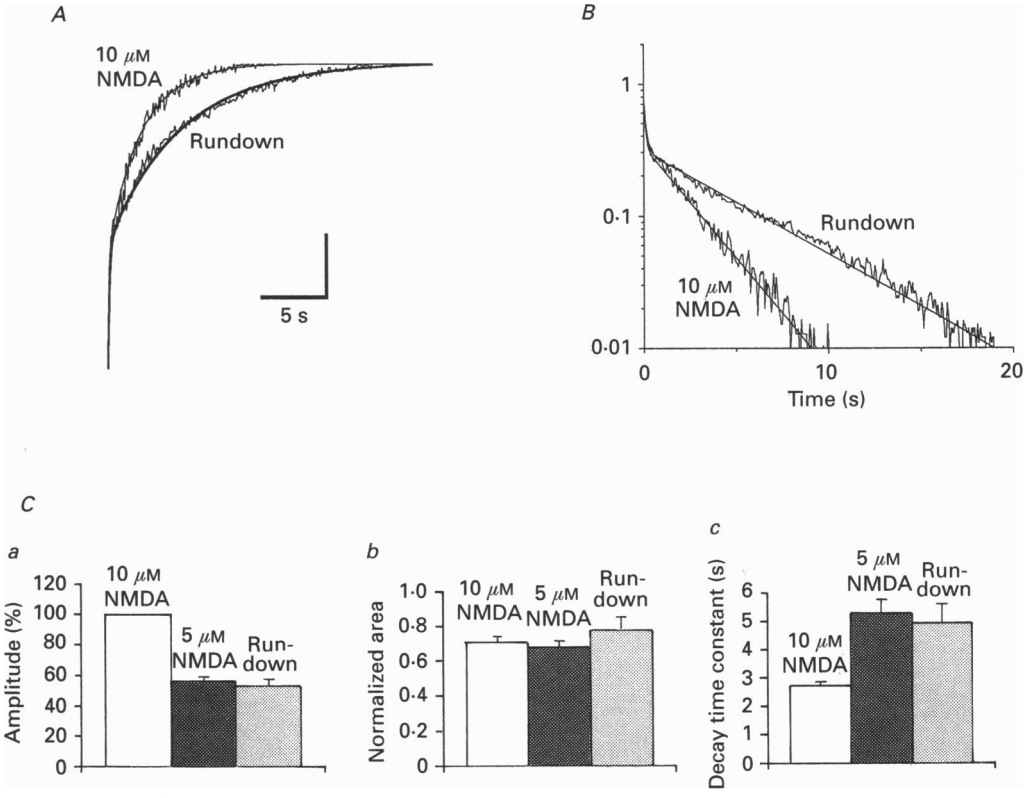

Fig. 10. Rundown resulted from a reduction of the open probability. *A*, the decay of the current following solution exchange from 10 μM NMDA to NMDA +20 μM MK-801 are superimposed for a neuron before rundown ('10 μM NMDA') and for a second neuron after rundown ('Rundown'). The currents were normalized to the steady-state current immediately preceding exposure to MK-801; calibration was 130 and 70 pA, respectively. The best fit to the sum of two exponentials is shown; the fast time constant was unchanged following rundown (see text). The slow time constant was 2·49 s (coefficient 30·6%) before rundown and 5·39 s (coefficient 31·5%) after rundown for these two neurons. *B*, semilogarithmic plot of the current decays in MK-801 with superimposed exponential curve fits are shown for the neurons in *A*. *C*, histograms of the steady-state current amplitudes (*a*), charge transfer during MK-801 block (expressed as nC per unit steady-state current of 1 nA, *b* and the slow time constant (*c*) are shown for low (5 μM) and high (10 μM) concentrations of NMDA and following rundown in 10 μM NMDA. The steady-state current amplitudes, normalized to the current evoked by 10 μM NMDA, were 56·2% ± 1·6 (5 μM NMDA, $n = 17$) and 53·5 ± 3·5, $n = 6$ (10 μM NMDA after rundown). The charge transfer was 0·71 ± 0·03 nC (10 μM NMDA, $n = 6$), 0·68 ± 0·03 nC (5 μM NMDA, $n = 5$) and 0·78 ± 0·08 nC (10 μM NMDA after rundown, $n = 6$). The slow time constant for 10 μM NMDA was 2·74 ± 0·08 s (coefficient 31·1 ± 2·3%) which was significantly faster than for 5 μM NMDA (5·3 ± 0·5 s (coefficient 30·8 ± 1·9%, $P = 0·007$) or following rundown (5·0 ± 0·7 s, coefficient 32·3 ± 1·2%, $P = 0·003$).

summarized in Fig. 10*C*. Thus a change in P_o is sufficient to account for rundown of the NMDA channel.

DISCUSSION

Our results suggest that the rundown of NMDA channels requires transmembrane calcium entry and results from a reduction in the probability of channel opening. Rundown was incomplete, never exceeding around 50% during recording periods of up to 45 min. As reported previously, ATP regenerating solution was effective in preventing rundown, but appeared to involve mechanisms other than direct receptor phosphorylation. ATP was only required in the presence of ongoing calcium entry.

Comparison to previous results

Rundown of ion channel activity has been a common observation, particularly since the advent of whole-cell recording (Byerly & Hagiwara, 1982; Fenwick *et al.* 1982). However, multiple mechanisms appear to be involved in this behaviour. Thus evaluation of rundown is sensitive to cell and recording conditions, particularly access resistance, voltage- and space-clamp and the use of a standard protocol to evaluate use and time dependence. For example, alternating applications of kainic acid in Ca^{2+}-containing solutions and NMDA in Ca^{2+}-free solutions did not lead to rundown of the NMDA channel (not shown). If voltage escape had occurred, Ca^{2+} loading during kainic acid applications should have caused rundown. We also closely monitored access resistance and used concentration clamp methods that allowed control of agonist applications. The adequacy of dialysis was facilitated by the use of large-tipped pipettes and dialysis rate was measured using a low M_r test reagent (*N*-methylglucamine, M_r 195, see Methods). Based on this rate (e.g. 90 s complete exchange time for *N*-methylglucamine), we further delayed the beginning of each experiment (2–3 min) to allow diffusion of the higher M_r buffers and test reagents into the cell. We also used low concentrations of NMDA ($< EC_{50}$) and saturating concentrations of glycine so that rundown could be studied without contamination by glycine-dependent and -independent desensitization (Mayer, Vyklicky & Clements, 1989; Sather *et al.* 1990).

For the NMDA channel, MacDonald *et al.* (1989) reported rapid washout of currents with a time constant of approximately 150 s that did not spontaneously recover. Washout was prevented by inclusion of an ATP regenerating solution in the pipette and was unaffected by changes in the dialysate calcium concentration or by substitution of BAPTA for EGTA in the pipette. Thus MacDonald *et al.* (1989) concluded that washout did not result from accumulation of intracellular calcium and was likely due to phosphorylation of the NMDA receptor or a related protein. The characteristics of rundown in our experiments differed, although in both cases maximal rundown was approximately 50%. Rundown was slower and reversible under our conditions. However, we were able to induce rapid, irreversible rundown using high agonist concentrations and application frequencies. The most striking difference was the absolute Ca^{2+} dependence of rundown in our experiments. This was supported by several observations: the absence of rundown in Ca^{2+}-free medium; rapid rundown following block of the Na^+–Ca^{2+} exchanger; inhibition of NMDA current by intracellular perfusion with high [Ca^{2+}] and the use dependence of rundown in the presence of extracellular calcium. High concentrations of BAPTA

were effective in preventing rundown, but this could be overcome by large transmembrane calcium fluxes generated by high agonist concentrations or frequent NMDA applications. Our experiments might be reconciled with those of MacDonald *et al.* (1989) by assuming that brief calcium transients rather than accumulation of intracellular calcium are the trigger for rundown, although we cannot exclude that separate mechanisms may be involved.

Washout of soluble factors can be a contributing factor to channel rundown (Byerly & Hagiwara, 1982; Fenwick *et al.* 1982; Chen *et al.* 1990). However, in our experiments no apparent loss of activity was observed if we waited up to 15 min before the start of agonist applications in Ca^{2+}-containing solutions, suggesting that receptor activation followed by Ca^{2+} entry was the trigger required to initiate rundown.

Calcium and NMDA receptor activity

Several effects of calcium on NMDA channel activity have been reported. Although NMDA channels are Ca^{2+}-permeable, extracellular calcium reduces the single channel conductance (Jahr & Stevens, 1987; Ascher & Nowak, 1988). In addition, decreases in NMDA current have been reported following procedures that increase intracellular calcium (Mayer & Westbrook, 1985; Mayer, MacDermott, Westbrook, Smith & Barker, 1987; Zorumski, Yang & Fishbach, 1989). However it was unclear whether this simply reflected Ca^{2+} dependence of receptor desensitization. More recently, several distinct forms of NMDA receptor desensitization have been characterized including glycine-dependent desensitization (Mayer *et al.* 1989) and a rapid glycine-independent desensitization that is particularly prominent in outside-out patches (Sather *et al.* 1990). Neither of these mechanisms require calcium. Modulation of voltage-dependent desensitization of NMDA currents by $[Ca^{2+}]_o$ has been reported (Clark *et al.* 1990; Zilberter, Uteshev, Sokolova & Motin, 1990), but it is unclear if this action of calcium is intracellular or extracellular. We recently found that increases in $[Ca^{2+}]_i$ underlie the slow fade ('inactivation') NMDA currents during agonist applications lasting 5–15 s (Legendre *et al.* 1993). The whole-cell current decreased by 50% with a time constant of approximately 1–5 s that was dependent on $[Ca^{2+}]_o$ and the concentration of EGTA in the pipette. However, unlike rundown, inactivation was not prevented by ATP and barium could substitute for calcium. Thus calcium is likely to have more than one effect in the regulation of NMDA channel activity.

Rundown was highly use-dependent in our experiments. Thus rundown could conceivably result either from gradual increases in the steady-state $[Ca^{2+}]_i$, or from the cumulative effects of multiple calcium transients. The steady-state Ca^{2+} concentration should have been well controlled by the exogenous calcium buffer. However, EGTA was less effective in preventing rundown than the more rapid buffer BAPTA suggesting that the amplitude and duration of the calcium transient were important factors (Adler *et al.* 1991). This is consistent with recent studies demonstrating that Ca^{2+} may reach concentrations as high as 1 mM in the submembrane compartment even in the presence of EGTA (e.g. Augustine & Neher, 1992).

Site of action of intracellular calcium

Our results strongly suggest that the Ca^{2+} and ATP dependence of rundown are linked. In particular, ATP regenerating solution had no effect on the response in the absence of calcium entry. Likewise, rundown induced by calcium entry through NMDA channels was overcome by perfusion with ATP regenerating solution. As it has been proposed that phosphorylation is necessary for maximal channel activity (MacDonald *et al.* 1989), one simple hypothesis might be that Ca^{2+} acts on a phosphatase to induce rundown. This has been proposed for the Ca^{2+}- and ATP-dependent rundown of $GABA_A$ receptors (Chen *et al.* 1990, but see Shirasaki, Aibara & Akaike, 1992). However, no reduction in NMDA currents was observed following dialysis with several phosphatases and rundown was not prevented by phosphatase inhibitors, the activity of which were verified *in vitro*. There is also evidence that NMDA receptor stimulation can activate Ca^{2+}-dependent proteases (Siman & Noszek, 1988). However, the reversibility of rundown also is evidence against a primary action of a Ca^{2+}-dependent protease. Although rundown became irreversible following large calcium loads, this was unaffected by dialysis with protease inhibitors.

Rundown did not occur in barium solutions and was reduced by calmidazolium, suggesting that Ca^{2+} acts on a calmodulin-dependent protein. Further experiments will be required to determine which CaM-dependent proteins contribute to NMDA channel rundown. However, we have not observed any Ca^{2+}-dependence of NMDA channels in inside-out patches (C. Rosenmund, unpublished), perhaps suggesting that the calcium binding site involves a regulatory component that is lost in isolated membrane patches.

How does ATP maintain the NMDA response?

Kinase stimulation has been reported to potentiate NMDA responses (Chen & Huang, 1991). Although ATP regenerating solution prevented NMDA channel rundown, our experiments suggest that this is not primarily due to an action as a kinase substrate. In particular, the high concentrations of ATP necessary for maintenance of the NMDA response and the low efficacy of ATP-γ-S in our experiments are inconsistent with that expected for maximal stimulation of kinases. In the presence of ATP regenerating solution, we did observe a slight rundown in the presence of high concentrations of staurosporine, thus we cannot exclude the possibility that phosphorylation of a regulatory protein contributes to maintenance of the current. However, this could also be non-specific, e.g. kinase inhibitors can have direct effects on channel activity including NMDA channels (e.g. Amador & Dani, 1991).

An alternative mechanism for the action of ATP regenerating solution is to facilitate the clearance of intracellular calcium from the cytosol. In neurons, clearance of cytoplasmic calcium is controlled primarily by the Na^+–Ca^{2+} exchanger (Blaustein, 1988; Miller, 1991). ATP modulates the function of the Na^+–Ca^{2+} exchanger, and functional Na^+–Ca^{2+} exchange was required to prevent rundown in the presence of ATP regenerating solution. In squid axons, ATP (dissociation constant, $K_d \approx 250\ \mu M$) increases the affinity of Ca^{2+} for the exchanger, presumably

via phosphorylation (Blaustein, 1977; Miller, 1991). In addition, high concentrations of ATP ($EC_{50} \approx 3$ mM) stimulate the exchanger in cardiac myocytes (Collins, Somlyo & Hilgemann, 1992). This may result from stimulation of aminophospholipid translocase (Hilgemann & Collins, 1992), an enzyme that maintains the asymmetry of membrane lipids and thus regulates the association of proteins with the membrane (Devaux, 1991).

Functional consequences

Our results indicate that the open probability of NMDA channels is regulated by receptor-mediated calcium influx. Although the calcium influx associated with long applications of agonist in whole-cell recording is large, under physiological conditions, Ca^{2+} can reach quite high levels in localized cytosolic compartments (Augustine & Neher, 1992), particularly during periods of intense synaptic activity. This places a premium on the adequacy of calcium homeostasis in dendritic spines (e.g. Zador, Koch & Brown, 1990). Otherwise rundown of NMDA channel activity will occur and the neuromodulatory influence of these receptors on synaptic transmission will be diminished.

Note added in proof. We have recently found that calcium and ATP can regulate activity of NMDA channels by altering the polymerization of the actin cytoskeleton (Rosenmund & Westbrook, 1993).

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