INTRACELLULAR REGULATION OF THE NMDA CHANNEL

by

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ABSTRACT

Synaptic activation of N-methyl-D aspartate (NMDA) channel in the vertebrate brain induces an elevation of intracellular calcium in the postsynaptic spine of central neurons. Although this step is essential for the induction of some forms of development and learning, the sites of action in the spine are not known. Proposed actions of calcium are numerous including gene regulation, activation of kinases or phosphatases as well as involvement of other calcium-dependent proteins, and allosteric modulation of membrane receptors. The purpose of this study was to examine the feedback regulation of NMDA channels by intracellular calcium and other regulatory factors. Standard whole-cell and single channel patch-clamp recordings on primary cultures of neonatal rat hippocampi were employed. The cytosol was controlled by whole-cell dialysis and perfusion techniques; and the extracellular medium was altered using rapid perfusion.

Two forms of channel modulation were studied: calcium-dependent inactivation and channel rundown. During whole-cell or cell-attached recording, the channel underwent partial inactivation upon elevation of intracellular calcium. Channels studied in isolated patches lost their calcium sensitivity. Recovery from inactivation was dependent on the presence of intracellular ATP. Rundown of channel activity during prolonged whole-cell recording was also dependent on intracellular calcium, and was blocked by addition of ATP to the recording pipette.

Possible enzymatic mechanisms underlying the action of calcium and ATP were studied. Manipulation of intracellular phosphorylation / dephosphorylation resulted in mostly insignificant effects. One common target of calcium and ATP in channel rundown was the actin cytoskeleton. Rundown, but not Ca-dependent inactivation, was blocked by phalloidin, a toxin that inhibits actin depolymerization. Cytochalasins depolymerize actin filaments by enhancing the hydrolysis of the ATP-actin complex. They antagonized the effect of ATP in our experiments, suggesting that ATP maintains filamentous actin.

These data suggest that regulation of NMDA channels by intracellular calcium involves two distinct mechanisms. One is mediated by depolymerization of actin, suggesting that the cortical cytoskeleton is necessary for the maintenance of channel activity. The second effect, calcium-dependent inactivation, existed only in intact cells and was not blocked by intracellular ATP or phalloidin. A model for channel regulation that includes a calcium-dependent regulatory subunit held in place by the actin cytoskeleton can account for the observations. The functional interaction between the neuronal cytoskeleton and the activity of the NMDA channel provides further evidence for a dynamic interaction of membrane proteins and the structural elements of the neuron.

INTRODUCTION

Membrane ion channels are the principle determinants of electrical excitability. The regulation of ion channel activity is therefore of fundamental importance in understanding signaling in the nervous system. The subject of this dissertation is the cellular elements that regulate the L-glutamate gated N-methyl-D-aspartate (NMDA) channel. The NMDA channel participates in long-term changes in synaptic activity, such as in neural development and in long-term potentiation (LTP), an experimental model of memory. Consistent with this functions, the NMDA channel appears to be highly regulated by a variety of mechanisms. For example, it is known that the permeation of calcium through the channel is the trigger for long-term changes in synaptic efficacy, it is unknown what effect the resulting cytosolic calcium elevation has on the NMDA channel function itself. Early reports claimed an inhibitory effect of intracellular elevation of calcium $[Ca]_i$, however recent reports challenged these findings. The experiments reported here were designed to characterize the regulation of the NMDA channel by calcium and other factors and to identify the mechanisms underlying this feedback regulation.

The introduction reviews the physiology of the NMDA channel, cellular mechanisms of calcium homeostasis, and the potential enzymatic targets of calcium. Particular emphasis will be placed on the actin cytoskeleton. Important principles of channel modulation will be discussed in terms of previous studies of the voltage-dependent calcium channel and the nicotinic acetylcholine receptor. Finally the modulation of glutamate channels is reviewed.

THE PHYSIOLOGY OF THE NMDA CHANNEL

Presynaptic release of L-glutamate excites neurons by opening cation channels.

Glutamatergic neurotransmission is the most common form of excitatory synaptic projection in the central nervous system (Mayer and Westbrook, 1987a; Westbrook and Jahr, 1989). As an action potential reaches the presynaptic terminal of an excitatory neuron, L-glutamate containing vesicles release their contents into the synaptic cleft. Glutamate molecules diffuse to the postsynaptic membrane, bind to glutamate-gated channels that in turn open cation conducting pores. The resulting excitatory postsynaptic current (EPSC) is based on the coactivation of two classes of channels (Forsythe and Westbrook, 1988; Bekkers and Stevens, 1989). These glutamate channel subtypes can be separately activated by the agonists α-amino-3-hydroxyl-5-methyl-4-isoxalone propionic acid (AMPA) and NMDA. An additional class of glutamate receptors, metabotropic receptors, are G-protein coupled (Nakanishi, 1992).

The two classes of glutamate-gated channels can not only be activated by different agonists, they differ in various functional properties. The AMPA channel mediates the current flow of Na^+ and K^+ ions, just as the nicotinic acetylcholine receptor (AChR) at the neuromuscular junction (Mayer and Westbrook, 1987b; Takeuchi and Takeuchi, 1960). The low affinity of the channels for their endogenous neurotransmitters produces in both cases rapidly mediated currents (Magleby and Stevens, 1972; Finkel and Redman, 1983; Patneau and Mayer, 1990; Silver et al., 1992). AMPA mediated synaptic

responses are therefore analogous to muscle AChR and are primed for 'normal' cell-to-cell communication.

The response mediated by the NMDA channel differs from the non-NMDA response in several aspects: voltage dependence, kinetics, and ion permeability. When the cell is near its resting membrane potential, the divalent cation magnesium in the extracellular space blocks the pore of the NMDA channel. Magnesium leaves the pore during significant postsynaptic depolarization as may occur during strong excitation (Nowak et al., 1984; Mayer et al., 1984). Thus NMDA channels are voltage-dependent and contribute more to the synaptic current when the membrane potential is depolarized.

Second, NMDA mediated currents exhibit unusually slow activation and inactivation time courses (Forsythe and Westbrook, 1988; Lester et al., 1990; Hestrin et al., 1990). For example, NMDA synaptic currents of cerebellar granule cells reach their maximum well after the AMPA mediated component has decayed (Silver et al., 1992). NMDA receptor mediated synaptic currents decay biphasically, with time constants of around 70 and 250 ms (20° C, e.g. Lester et al., 1990; Lester and Jahr, 1992), that may vary during development (Hestrin, 1992). The resulting currents, lasting for hundreds of milliseconds, can facilitate temporal summation of mono- and heterosynaptic input.

Third, the NMDA channel is highly permeable to calcium, with approximately 15 % of the charge is carried by the divalent cation (Mayer and Westbrook, 1987b). Intracellular calcium is an important second messenger

that can activate multiple metabolic and structural changes, with numerous potential consequences for neuronal behavior.

The molecular structure of the NMDA channel. By analogy with the AMPA channel and other ligand gated ion channels (Gasic and Heinemann, 1991; Betz, 1990), the NMDA channel is presumably a heteropolymer of subunits each with four transmembrane segments. Several subunits have been cloned (Moriyoshi et al., 1991; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992), and it is likely that NMDA receptors in native cells represent combination of heterogeneous subunits. The initially cloned NMDAR-1 subunit (Moriyoshi et al., 1991) shows only weak current expression, compared to combined expression of NMDAR-1 with any of the NMDAR 2 (a-d) subunits (Meguro et al., 1992; Monyer et al., 1992; Nakanishi, 1992). Structure-function studies have not yet identified the binding sites of glutamate and the coagonist L-glycine (Johnson and Ascher, 1987). The putative second transmembrane spanning region of NMDAR-1,2's contains an asparagine that appears to be responsible for regulating the calcium conductance and the voltage-dependent Mg^{2+} block (Burnashev et al., 1992; Mori et al., 1992). The cytoplasmic loops of the channel subunits also contain several consensus phosphorylation sites. The NMDAR-2a,b subunits contain a double repeat of an unusual pseudo zinc finger motif in one of the cytoplasmic loops (Monyer et al., 1992). One could speculate that this structure is involved in the interaction of divalents and/or other proteins. This site could be relevant to the results presented in this dissertation. We propose

that the NMDA channel is modulated in its gating by an interaction with calcium and a putative regulatory protein (see manuscript III).

The NMDA channel as mediator of plasticity

An essential function of higher neuronal processing is learning. Hebb (1949) postulated that learning occurs by an increase in the efficiency of synaptic transmission at single synapses. A corollary feature of the Hebbian synapse is that the process of synaptic strengthening should be use-dependent, requiring concurrent pre-and postsynaptic events. The unusual biophysical properties of the NMDA channel (voltage-dependence and calcium permeability) are ideally designed to serve as the molecular entity for the Hebbian synaptic learning event. The channel serves as a molecular switch that can translate strong excitatory input into a calcium signal in the postsynaptic spine. Assuming that calcium elevation is the second messenger that induces the changes in synaptic strength, this mechanism fulfills the requirement for a Hebbian form of learning. The following section introduce some evidence for Hebbian learning during neuronal development and in hippocampus of the adult rat.

Neuronal development. In the visual system, the involvement of NMDA receptors has been identified in early and late phases of development including the use-dependent modification of inputs to the visual cortex (Constantine-Paton et al., 1990), stabilization of functional connections, ocular dominance plasticity (Ranschecker and Hahn, 1987; Kleinschmidt et al., 1987) as well as the elimination of supernumerary inputs in later phases of visual

development (Collingridge and Singer, 1990). In addition, the formation of neural topographic maps in the frog as well as in the mammalian visual system has been shown to be dependent on NMDA receptors (Simon et al., 1992).

In the cerebellum, the elimination of supernumerary inputs results in 1:1 mapping of single climbing fibers to individual Purkinje neurons. NMDA antagonists block this synapse elimination (Rabacchi et al, 1992), suggesting the involvement of the NMDA channel. Consistent with such a developmental role, Purkinje cells transiently express NMDA channels during that period (Dupont et al., 1987; Rosenmund et al., 1992).

Long-term potentiation(LTP). LTP is a cellular phenomenon describing a long-lasting increase in synaptic efficacy at excitatory synapses (Bliss and Lomo, 1973; Madison et al., 1991). It is the most widely studied cellular model for learning and memory. Although LTP occurs in many central and peripheral synapses, LTP has been most intensively studied in the hippocampus, a region of the allocortex that is strongly implicated in learning events. In slice preparations, LTP can be produced by a short train of high-frequency stimulation of the presynaptic projection to the CA1 region. The involvement of the NMDA channel in the induction of LTP has been shown in various preparations (Collingridge et al., 1983; Morris et al., 1986), but not in the mossy fiber-CA3 pathway (Harris and Cotman, 1986). As a requirement for LTP, the role of the NMDA channel was proposed as: " LTP will only occur when a critical level of current flows through the NMDA receptor channel in the postsynaptic cell" (Collingridge and Bliss, 1987; Gustafsson and

Wigström, 1988). The critical component of that current is calcium. Lynch and colleagues (1983) were the first to demonstrate that preventing the postsynaptic rise of calcium blocked LTP. Accordingly, the release of postsynaptic calcium by photolysis of caged calcium buffer has been shown to mimic LTP (Malenka et al., 1988). However, the elevation of calcium through calcium channels on dendrites only transiently potentiates excitatory synaptic transmission, suggesting that calcium entering via NMDA and calcium channels produce segregated calcium signals (Regehr et al., 1989; Müller and Conner, 1991; Regehr and Tank, 1992; Kullmann et al., 1992).

The targets of calcium are yet unknown, although there is no lack of candidates. An early hypothesis for the mechanism of LTP proposed that the transient elevation of calcium activates proteases such as calpain. Fodrin (brain spectrin) is a good substrate for this protease and is a major component of the cortical cytoskeleton (Siman and Noszek, 1988). Limited proteolysis was proposed as a mechanism leading to an increase in the insertion of glutamate receptors in the postsynaptic membrane (Lynch and Baudry, 1987). Although the details of this 'postsynaptic' hypothesis of LTP, i. e. an increased number or sensitivity of postsynaptic receptors are no longer considered likely, evidence for postsynaptic factors in the maintenance of LTP exists (Kullmann et al., 1992).

More direct evidence exists for the involvement of protein kinases in the induction of LTP. Specific inhibitors of protein kinase C as well as CaM kinase II blocked LTP (Malinow et al, 1989; Malenka et al., 1989). Similar results have been obtained with less specific reagents (Lovinger et al., 1987; Malinow et al., 1988). However, the specific role of kinases in LTP is unclear

as their target(s) are yet unidentified. In addition, the persistency of LTP over hours *in vitro* and weeks *in vivo* would require persistent kinase activation, suggesting that other factors such as changes in spine structure, gene expression, or protein content could be involved (Fifkova, 1985; Frey et al., 1988; Cole et al., 1989).

Although it is generally agreed that induction of LTP occurs on the postsynaptic site, whether the maintenance phase involves pre- or postsynaptic mechanisms requires clarification (e.g. Bekkers and Stevens, 1990; Manabe et al., 1992). Postsynaptic localization might result from a change in the number or efficacy of postsynaptic glutamate channels. Studies of AMPA and NMDA channel regulation are important in assessing their involvement in the postsynaptic maintenance of LTP.

Long-term depression (LTD). Activity-dependent inhibition of excitatory synapses have been reported in cerebellar, cortical and hippocampal regions (Ito, 1989; Artola et al., 1990; Dudek and Bear, 1992). The involvement of NMDA channels in some cases are established. For example, blockade of NMDA channels with the competitive antagonist DL-2-amino-5-phosphonovalerate (DL-AP5) (Dudek and Bear, 1992) or injection of calcium-chelators (Artola et al., 1992; Mulkey and Malenka, 1992) inhibited the development of LTD, suggesting that NMDA mediated calcium entry is responsible. Interestingly the activity-dependent variations - 100 Hz stimulation for 1s in LTP and 1 Hz stimulation for 10 minutes in LTD - can lead to opposite changes in synaptic efficacy, presumably by acting on distinct calcium-dependent effector systems with different "thresholds".

Excitotoxicity

Glutamate-mediated neurotoxicity has been implicated in numerous acute and chronic diseases (Choi, 1988). Studies in experimental models of stroke and epilepsy demonstrate glutamate can mediate cell death; but glutamate may also be involved in the etiology of slowly progressing neurodegenerative diseases such as Huntington's disease. for example, a recent study has suggested that impaired reuptake of glutamate could contribute to the death of motor neurons in amyothrophic lateral sclerosis (Rothstein et al., 1992). Pharmacological studies suggest that excessive NMDA as well as non-NMDA receptor stimulation kills neurons (Rothman and Olney, 1986; Murphy et al., 1987; Choi et al., 1988). The NMDA channel appears to be specifically responsible for delayed excitotoxicity in *in vitro* models. Other mechanisms that raises $[Ca]_i$ excessively or chronically, mimic the effect of NMDA activation (Nachshen et al., 1986), suggesting that excitotoxicity is mediated via calcium influx into neurons.

The precise mechanism of calcium is not known. However, the long-term elevation of calcium may favor chronic stimulation of catabolic enzyme cascades, resulting in the depletion of adenosine-5-triphosphate (ATP, Cheung, 1986). Ca^{2+} may also cause the activation of proteases such as calpain, resulting in excessive proteolytic reactions (Siman and Noszek, 1988). Calcium-sensitive phospholipases may affect membrane function by hydrolysis of phospholipids as well as though excessive production of oxygen radicals (Chan and Fishman, 1978; Dykens et al., 1987).

A different mechanism of calcium-mediated neurotoxicity may occur via the production of nitric oxide (NO). Synder and colleagues showed that the NMDA-mediated neurotoxicity in neuronal cultures is significantly reduced by inhibitors of NO synthesis (Dawson et al., 1991; Bredt and Snyder, 1992). In related experiments, the NO production was associated with neuronal cell death during focal ischemia in a rodent stroke model (Nowicki et al., 1991). These observations demonstrate that uncontrolled and long-lasting elevation of intracellular calcium can have dramatic effects on neuronal tissue, thus it is not surprising that intracellular calcium is tightly regulated.

CALCIUM HOMEOSTASIS

Our results demonstrate that intracellular calcium induces inactivation and rundown of NMDA channels. To understand the physiological significance of this observation, it is important to consider the various factor contributing to the calcium homeostasis.

Calcium as an intracellular signal

Transient elevation of $[Ca^{2+}]_i$ is an important signal for many neuronal functions, ranging from transmitter release, growth cone regulation, long term potentiation and neuronal cell death. The unique behavior of the physiologically ubiquitous divalent calcium is to reversibly chelate with proteins with association constants of high nanomolar to low micromolar.

The binding of calcium can result in conformational changes in the protein and therefore alter its function. Thus transient elevations of calcium serves as an intracellular signal. Active transport by ATPases and exchanger extrudes calcium out of the cell against a concentration gradient of about 10,000 fold. Just as the Na/K plasma membrane gradient is generated by the Na/K ATPase to provide the energy for electrical signals, the calcium gradient serves to enable biochemical signals. The control of cytoplasmic calcium levels is not only important in determining the $[Ca]_i$, the location and kinetics of extrusion mechanisms also determines the shape of the calcium transient. The first factor that determine the shape of the calcium signal is the source of calcium influx. The return of the elevated calcium to its basal level is controlled by intracellular buffers and extrusion mechanisms.

Calcium influx

The voltage and concentration gradient across the neuronal plasmalemma provide a strong driving force for calcium. Most calcium enters through calcium-permeable channels, such as voltage-gated calcium channels; and ligand gated cationic channels such as the NMDA channel. Other sources of calcium influx may come through nonspecific membrane "leak", or as Blaustein (1988) proposed, through the Na/Ca exchanger during action potentials (Leblanc and Hume, 1990). Voltage dependent calcium channels are ubiquitously distributed, with clustered patterns in dendrites (Westenbroek et al., 1992). Their contribution to the influx depends mainly on the level of excitation of the neuron, e.g. the rate and length of action potentials. Calcium

flux through NMDA channel is dependent on the membrane depolarization that relieves the Mg-block, as well as on glutamatergic synaptic input.

Intracellular calcium buffering and uptake

Intracellular buffering is a powerful mechanism for controlling cytosolic free calcium concentration. Although the steady-state calcium concentration in neurons is in the range of 100 nM, the total calcium concentration is in the range of several tens of micromolar (McBurney and Neering, 1987; Miller, 1991). This implies that there is a system of buffering and uptake that protects the neuron from toxic levels of calcium. Calcium buffers can be categorized into three groups: mitochondria, the endoplasmic reticulum, and calcium binding proteins. Although there is accumulating evidence for the physiological role of the calcium buffers, it is not clear whether these mechanism are operative in neurons dialyzed with external Ca^{2+} buffers in whole-cell recordings.

Calcium binding proteins. Neuronal cytosolic proteins such as calmodulin, parvalbumin and calbindin can bind calcium with affinities of high nanomolar to micromolar, and are present in quantities sufficient to provide significant buffer capability (Heizmann and Bechtolt, 1987; Carafoli, 1987; Neher and Augustine, 1992). Calmodulin also serves as a calcium receptor. Numerous regulatory enzymes including kinases, phosphatases, ATPases and cytoskeletal proteins are activated or modulated by the calcium-calmodulin complex (Heizmann and Berchtold, 1987; Cohen and Klee, 1988).

Calcium binding proteins have a predictable influence on the buffering of calcium, however data supporting a physiological role are sparse. For example, in bovine chromaffin cells estimates of the fate of calcium after entry through calcium channels was measured (Neher and Augustine, 1992). The endogenous buffer was immobile (equivalent to MW of 10^7 D), of low affinity (5-10 μM), and was present in relative high concentrations (350-700 μM). As a result, 98-99 % of the entering calcium, was buffered. An interesting observation has been made by Baimbridge and colleagues (1985). They found a depletion of calbindin in dentate gyrus granule cells following chronic epileptiform activity, and proposed that this depletion led to the altered excitability.

Mitochondria Mitochondria can sequester large quantities of calcium. The proton gradient created by the respiratory chain normally is used to synthesize ATP. However, under calcium loads the driving force can be entirely diverted to sequester cytosolic calcium (McCormack and Denton, 1988). Uptake and release rates are equal at the 'set-point', which is at around 1 μM. Thus, the physiological role of mitochondria in regulation of calcium would be significant only in regions with large calcium transients, such as in the nerve terminal and in the postsynaptic spine. For example, Delaney and colleagues compared the presynaptic $[Ca]_i$ time course to the potentiation of postsynaptic potential in the neuromuscular junctions of crayfish. The time of potentiation was similar to the time presynaptic [Ca]i was held at the mitochondrial 'set-point', suggesting a buffering role of mitochondria underlying this phenomenon (Delaney et al., 1989).

Endoplasmic reticulum (ER). Neurons contain non-mitochondrial organelles that can sequester and release calcium at physiological cytosolic calcium concentrations. They are anatomically identical to the endoplasmic reticulum and are found throughout neuronal compartments (Henkart et al., 1978; Miller, 1991). The functional properties of uptake, storage and release are comparable to the sarcoplasmatic reticulum (Blaustein et al., 1978). The sequestration into the ER is ATP dependent. Structurally and functionally the ER associated ATPase is closely related to the Ca/H ATPase found in the plasmalemma, except that the ER ATPase is calmodulin-insensitive (McGray et al., 1980; Carafoli, 1987). The physiological role of the ER related calcium uptake is different than mitochondria. The ER is not capable of buffering larger calcium loads, as it possesses a low sequestration capacity. However, the ER-ATPase 'fine-tunes' the cytosolic $[Ca]_i$ to physiological levels due to an affinity to cytosolic calcium of approximately 200 nM (Rasgado-Flores and Blaustein, 1987).

Calcium extrusion

Long-term stability of cytosolic calcium levels require that calcium influx and efflux are balanced. Neurons posses two parallel but independent mechanisms for extrusion: the Na/Ca exchanger and the plasma membrane calcium pump. The two extruders differ in their transport capacity and in their range of activation.

The Na/Ca exchanger The Na/Ca exchanger does not directly utilize chemical energy, it extrudes calcium by utilizing the sodium gradient. The driving force of the exchanger depends on the reversal potentials of sodium and calcium. Transport capacity increases with hyperpolarized potentials (Carafoli, 1987). Flux measurements in synaptosomes predict an exchange of three extracellular sodium ions for one intracellular calcium (Sanchez-Armass and Blaustein, 1987), resulting in a net inward current. The Na/Ca exchanger can serve as a high capacity calcium extrusion system. The exchanger has a relatively low affinity to calcium, yet with the advantage of high turnover. This implies a physiological role that is restricted to the clearance of larger calcium transients.

The exchanger activity is regulated by $[ATP]_i$, presumably via a phosphorylation mechanism and with half-maximal activation at 250 μM (Blaustein, 1977; Baker and DiPolo, 1984). Hilgemann and colleagues proposed an additional mechanism for ATP (Collins et al., 1992). A kinase independent, ATP-induced activity was identified, with a half-maximal concentration of 3 mM ATP. They proposed that the modulation of exchanger activity in cardiac myocytes may result from changes in membrane lipid composition induced by a phospholipid translocase (Hilgemann and Collins, 1992).

The physiological function of Na/Ca exchange in cardiac myocytes is well established, however its role in the nervous system is less clear. In squid axon and in brain synaptosomes, the exchanger is the major mechanism extruding the bulk calcium from the cytoplasm (Blaustein et al., 1978; Sanchez-Armass and Blaustein, 1984). In dorsal root ganglion neurons inhibition of the exchanger has been reported to have only a weak or no effect

on the calcium efflux. These results have been explained by the large volume-to-surface ratio of the soma of the sensory neurons as well as by the mitochondrial uptake (Thayer and Miller, 1990; Benham et al., 1992). Korn and Horn (1988) studied the effect of the exchanger on calcium-dependent rundown of voltage-dependent calcium channels and on the activation of calcium-activated conductance. Inhibition of the exchanger in whole-cell recordings but not in 'perforated' whole-cell recording accelerated the calcium channel rundown, suggesting that channel rundown is mainly the result of the pipette dialysis. Modest effects of the exchanger inhibition on the duration of calcium-activated chloride tail currents were also observed.

The plasma membrane calcium pump (PMCP). The PMCP, due to its transport properties, may be more important for the regulation of basal and somatic calcium levels (Carafoli, 1991). The affinity of calcium for the PMCP is near the resting cytoplasmic calcium levels, but the transport capacity is limited. The stochiometry of the transport is presumably one calcium for one ATP in exchange for at least one H^+ (Rasgado-Flores and Blaustein, 1987).

The Ca/H ATPase of the plasma membrane is highly regulated. To extrude calcium in the range of submicromolar concentration, it requires the interaction with Ca-calmodulin (Carafoli, 1987). In the presence of the Ca-calmodulin complex the affinity to calcium increases from 10-20 μM to about 0.5 μM. The regulation of the ATPase by acidic phospholipids, mild calpain proteolysis, and phosphorylation has also been reported (Carafoli, 1988; Miller, 1991).

Studies on the physiological impact of the transport are hampered by the lack of specific inhibitors. However, vanadate inhibits P-type ATPases by binding to the γ-phosphate binding pocket (Carafoli, 1987, 1988), and the calmodulin antagonist calmidazolium can be used to prevent the high affinity mode of the pump. Two studies employed these inhibitors to test the physiological impact of the ATPase in cultured dorsal root ganglion cells (Thayer and Miller, 1990; Benham et al., 1992). Benham and colleagues were able to show a role of the ATPase in the clearance of calcium loads, whereas Thayer and Miller demonstrated that only the mitochondria participated in the clearance of intracellular calcium.

Role of ATP in calcium kinetics

ATP has two general functions in calcium homeostasis . First, ATP provides the energy to transport calcium against a concentration and voltage gradient. Consequently, the disturbance of ATP synthesis will lead to reduced capability to extrude calcium. In the case of the Ca-ATPase, ATP hydrolysis is directly utilized to extrude calcium over the plasmalemma membrane. The driving force for the Na/Ca exchanger is provided by the sodium gradient, set up by the Na/K ATPase. Second, both the pump and the Na/Ca exchanger can be modulated by mechanisms involving ATP. The proposed enhancement of calcium clearance by phosphorylation may be an interesting feedback signal, as increased calcium levels can activate calcium-dependent kinases (Carafoli, 1988; Scott and Soderling, 1992).

Localization and time course of calcium transients.

The end product of influx, buffering, and extrusion is the size and shape of the calcium transient. The temporal and spatial characteristics of this transient determine what class of enzymes are activated and in particular, their location. The understanding of calcium as an intracellular messenger has been dramatically enhanced within the last few years by the advent of calcium imaging techniques. However, the size and shape of calcium-transients in subcellular compartments such as dendrites, dendritic spines, or in the presynaptic terminal remain difficult to assess. Time and spatial resolution of calcium imaging systems are hampered by the properties of the available calcium sensitive dyes, as well as by the optical resolution of microscopes (Augustine and Neher, 1992). The current data suggests that

calcium in dendrites and in presynaptic terminals rise and decay rapidly, and reach peak concentrations in the submillimolar range (Adler et al., 1991; Llinas et al., 1992). Hudspeth and colleagues used a physiological bioassay to estimate $[Ca]_i$ at release sites of hair cells. Calcium was measured by calcium activated K^+ channels that are clustered with calcium channels. The estimated local concentration was up to 1 mM $[Ca]_i$ (Roberts et al., 1990).

The apparent restriction of calcium to small domains (Simon and Llinas, 1985; Fogelson and Zucker, 1985) close to the calcium source was supported by a recent study by Stryer and colleagues (Allbritton et al., 1992). The diffusion of calcium in cell extracts from *Xenopus oocytes* was measured, with particular emphasis on the buffer properties. The diffusion coefficient (D) ranged from 13 to 65 $\mu m^2/s$, depending on the initial concentration. This compared to D = 370 $\mu m^2/s$ in aqueous solution. They concluded that the range of diffusion for calcium away from a point source is mostly under one micron, and is present for only tens to hundreds of microseconds.

The geometry of the compartment is an additional determinant of the fate of the calcium signal. Presynaptic neurotransmitter release requires large, short lasting calcium transients, with the minimal requirement of an opening calcium conducting channel within 0.5 μM to the target (Augustine and Neher, 1992; Allbritton et al., 1992). Rapid termination of the calcium signal presumably occurs by diffusion and by transmembrane clearance as the high surface-to-volume ratio in the terminal should provide a high density of calcium extruders. Alteration of gene expression appears to require moderate, longer lasting elevations of calcium in the large somatic compartment (Cole et al., 1989; Morgan and Curran, 1989). It is predictable that

the underlying mechanism for the calcium signaling in the nucleus must include regenerating calcium signals, e.g. via internal stores (Berridge and Irvine, 1989; Miller, 1991), based on the limited diffusion capacity of free calcium.

Calcium in the postsynaptic spine. Dendritic spines are membrane protuberances in the range of roughly one micrometer in diameter and length (Harris and Stevens, 1989). Although these spines can have numerous shapes, the "average" spine of adult hippocampal pyramidal cells shows a spherical spine body connected to the main branch of the dendrite via a thin neck. It has been proposed that the synapse specificity of LTP to isolated inputs could be the result of the shape of the spine (Fifkova, 1985). Because of its geometry, elevation of calcium in the spine is thought to be isolated from the rest of the dendrite (Harris and Landis, 1986; Landis, 1988). This hypothesis has been supported by calcium imaging studies on cultured hippocampal neurons (Müller and Connor, 1991; Guthrie et al., 1991).

Models of calcium in the spine. Due to the experimental inaccessibility of the spine, the fate of calcium has been estimated by computer simulations (Gamble and Koch, 1987; Zador et al., 1990). These models, although limited in their quantitative accuracy by many free parameters, also suggest restricted transient changes in spine $[Ca]_i$ following synaptic input, and make several interesting qualitative predictions. Due to the small spine volume ionic fluxes should result in significant changes in ionic concentrations. The models also predict that peak $[Ca]_i$ in the spine can reach >10 μM, whereas the

dendrite calcium concentration remains at submicromolar concentrations. Clearance of calcium by extrusion should be faster than diffusional "escape" into the main dendritic branch, as the spine geometry with its high surface-to-volume ration provides a large transport capacity. This supports the idea of an calcium signal isolated to the excited spine. Furthermore, the model suggests that calcium transients can be long-lasting, based on the ongoing calcium entry during the slow decay of NMDA synaptic currents.

CALCIUM DYNAMICS

Calcium is central to the regulation of several classes of enzymes. In neurons, ion channels are one of the major targets for calcium. This form of modulation can occur via direct interaction of calcium with the channel protein, or it may involve specific calcium mediated second messenger systems such as calmodulin. Studies of ion channel regulation also suggest that phosphorylation mechanisms play a dominant role (Huganir and Greengard, 1991). Previous studies on the NMDA channel have suggested such phosphorylation-dependent regulation (see page 38). We also found that rundown of the channel is calcium- and ATP-dependent. Thus the following section will review the properties of multifunctional kinases and phosphatases as they relate to ion channel regulation. The second part will focus on an alternative target of intracellular calcium and ATP, the cytoskeleton.

Phosphorylation mechanism

Following the discovery of cyclic-adenosine-3',5'-monophosphate (cAMP) dependent phosphorylation in the regulation of glycogen metabolism (Krebs et al., 1959; Robinson et al., 1968), phosphorylation as a regulator of cellular processes has been widely recognized. For example, a large number of phosphoproteins play critical roles in the nervous system (Nestler and Greengard, 1984). Regulation by phosphorylation requires two groups of enzymes, kinases and phosphatases. Kinases catalyzing the transfer of gamma phosphates of ATP onto hydroxyl amino acids of the target protein. The transformation of hydroxyl group in a side chain of an protein into a negatively charged phosphate ester can have profound impact on the structure of the protein or its catalytic activity. A specific group of enzymes that hydrolyze these phosphate esters, phosphatases, provide the cell with a mechanism to reverse the action of the kinases.

A growing list of physiological reactions, from metabolic changes to alteration in gene expression, from neurotransmitter release to modification of membrane ion channels, are regulated by phosphorylation. Also, neuronal tissue contains large concentrations and diversities of enzymes involved in second messenger mediated phosphorylation responses (Scott and Soderling, 1992; Nairn and Shenolikar, 1992). Based on its impact, it comes as no surprise that kinases as well as phosphatases are under control of second messengers such as calcium and cAMP.

One could easily imagine how phosphorylation could alter channel function as outlined by Miller (1987). The addition of the phosphate group

results in spatial and electrostatic changes to the protein. Surface electrostatics have been shown to regulate gating of calcium activated K^+ channels (MacKinnon et al., 1989). Phosphorylation of proteins on the cytosolic surface could have a similar effect by increasing the density of negative charges. This has been probed in squid axons, resulting in a shift of the kinetic and conductive parameters of delayed rectifying potassium channels (Perozo and Bezanilla, 1990). Any structural modification that stabilizes or destabilize the 'open state' will alter the time the channel stays open or changes the opening probability following simple thermodynamic principles. The modulation by phosphorylation can occur on the channel itself or on proteins regulating channel function.

Kinases

The cAMP-dependent protein kinase (PKA) is the classic target enzyme for cAMP (Krebs et al., 1959; Sutherland, 1972). A neurotransmitter binds to a receptor on the nerve cell membrane. The ligand-receptor complex catalyzes the dissociation of G-protein heterotrimers and the activated alpha subunit then activates the enzyme adenylate cyclase to produce cAMP. The cyclic nucleotide binds to the regulatory subunit of the PKA holoenzyme and causes the release of the catalytic subunit and subsequent target phosphorylation. The activity of the kinase is in part regulated by mechanisms that specifically localize the holoenzymes in compartments. For example, the highest specific activity of the PKA has been found in the particulate fraction of synaptic preparations such as in the postsynaptic density (Kelly et al., 1979; Scott and Soderling, 1992). Protein kinase A has a broad range of substrates *in vitro*,

including several ion channels such as cardiac L-type calcium channels (Hartzell et al., 1991; Ono and Fozzard, 1992), γ-aminobutyric acid (GABA, Porter et al., 1990) and AMPA channels (Wang et al., 1991; Greengard et al., 1991).

Another class, the calcium/calmodulin dependent kinases (CaMK) are a family of isozymes that contain 6-12 subunits per holoenzyme (Bennett et al., 1983). Initiation of catalytic activity is dependent on the formation of an complex with Ca-CaM; thus this enzyme is directly dependent on calcium transients. Subsequent autophosphorylation can result in a calcium-independent kinase activity that can outlast the duration of the calcium transient. One subtype, CaMKII appears to be an important regulator of synaptic transmission. Large concentrations of CaMKII have been found in the postsynaptic density (Kennedy et al., 1983). Presynaptic function in the regulation of neurotransmitter release has been proposed. For example, injection of CaMKII into nerve terminals of the squid giant synapse altered the release of neurotransmitter, presumably via phosphorylation of synapsins (Llinas et al., 1991).

Protein kinase C (PKC) is activated by phospholipids such as diacylglycerol as well as by [Ca]*i* (Nishizuka, 1984). Some of the subtypes show specific tissue, regional and developmental expression patterns (Kikkawa et al., 1989). The subtypes can also differ in the sensitivity of their activators. For example, the γ-subtype, solely expressed in postnatal brain tissue, is less sensitive to diacylglycerol, but is significantly activated by arachidonic acid (Kikkawa et al., 1989). The role of PKC in mediating modulatory functions is substantiated by the fact, that all activators can be product of second

messenger pathways, thus being a second messenger -"responsive element". PKC undergoes translocation upon activation from the cytosol to membrane and cytoskeletal elements (Kraft and Anderson, 1983). The enzyme has a broad substrate specificity, and has been implicated in various physiological functions including ion channel regulation. However, not all of the functions of PKC are limited to the execution of second messenger signals, PKC actively participates in controlling the extent of calcium signals, as may it regulate the function of the Ca/H ATPase as well as the Na/Ca exchanger (Kikkawa et al., 1989).

Phosphatases

The cleavage of phosphate-protein esters is catalyzed by phosphatases . The four classes of phosphatases, Prp-1, Prp-2a, Prp-2b and Prp-2c have all been identified in the nervous system (Ingebritsen and Cohen, 1983; Nairn and Shenolikar, 1992). The regulation and localization of these phosphatases will influence the level of proteins that are phosphorylated. Protein phosphatase-1 (Prp-1) accounts for 25-40 % of the whole, and 80% of membrane associated phosphatase activity in the nervous system (Cohen et al., 1989). In the cytosol, this phosphatase is inhibited by endogenous proteins such as I-1, I-2 or DARPP-32 (Nairn and Shenolikar, 1992). Protein phosphatase type 2a (Prp-2a) is very abundant in the brain and represents 60-75% of the phosphatase activity (Cohen et al., 1989). The regulation and function of Prp-2a, however, is little understood.

The phosphatase type 2b, or calcineurin (CaN), is highly abundant in the brain (Ingebritsen and Cohen, 1983; Nairn and Shenolikar, 1992). It is the

only phosphatase known to be directly activated by calcium via an EF-hand type calcium binding site. Activation by the Ca-CaM complex, however, leads to a higher level of phosphatase activity than with calcium alone (Klee et al., 1979). The heterodimer complex is half maximally activated *in vitro* at around 1 μM $[Ca^{2+}]i$. The β-subunit was shown to undergo myristylation, suggesting that the CaN can be enriched at the membrane. Calcineurin dephosphorylates only a few substrates efficiently, but its physiological role in regulating calcium-dependent calcium-channel inactivation has been shown by Chad and Eckert (1986). To demonstrate the involvement of calcineurin in other processes, the available tools are indirect, including calmodulin inhibitors, calcium buffers and the immunosuppressant cyclosporine (Liu et al., 1991).

Proteases

Although there are variety of proteases in brain tissue, the most extensively investigated is the ubiquitously distributed calpain (Melloni and Pontremoli, 1989). The activity of calpain is tightly linked to cytosolic calcium. Calpain has a calmodulin-like structure and a micromolar affinity to calcium, resulting in the proteolytic activity. Multiple physiological functions of calpain have been proposed, as many proteins alter their function over partial proteolysis. For example, calpain can influence phosphorylation, as activated PKC is a good substrate (Melloni and Pontremoli, 1989). A major component of the cortical cytoskeleton, fodrin, is also proteolyzed by calpain (Siman and Noszek, 1988). This could be relevant to excitoxicity (Siman et al., 1989), but was initially proposed as a mechanism of long-term modification of synaptic efficacy by Lynch and colleagues (1987). They proposed that fodrin proteolysis

initiates a series of events leading to a putative increase of glutamate binding sites.

THE NEURONAL CYTOSKELETON

Neurons are filled with filamentous structures, mainly consisting of three cytosolic proteins: actin, neurofilaments and microtubuli. These molecules, generally classified as the cytoskeleton are responsible for structural functions such as the development and maintenance of the cellular morphology. Polymers are formed by intrinsic stereotypic mechanisms, but are also under the influence of extracellular signals. Examples for the dynamic interaction of the neuronal morphology and its environment is axonal growth cones (Forscher, 1989). Microtubuli are found in most parts of neurons and are involved in organizing axonal transport of macromolecules and organelles and are in the extension of the growing axon. Neurofilaments are similarly distributed as microtubuli. Although neurofilaments were the first filamental proteins known in neurons, their function remains poorly understood (Shaw, 1991). Most actin is found underneath the membrane, forming the cortical cytoskeleton.

The structure and organization of the dendrite and spine cytoskeleton are of particular interest in the regulation of postsynaptic receptors including NMDA channels. The postsynaptic density (PSD), a specialized structure underlying the postsynaptic spine, is a dense web of structural proteins that

contains second messenger regulated kinases, phosphatases and proteases (Gulley and Reese, 1981; Kennedy et al., 1983; Bamburg and Bernstein, 1991).

The actin cytoskeleton

Actin is the most abundant cytoskeletal protein in dendrites and particularly in the PSD (Matus et al., 1982). Actin is a bilobular-shaped 43 kD protein (G-actin) that can form homopolymers (F-actin). The central pocket formed by the two subunits contains the divalent cation (Ca^{2+} or Mg^{2+}) and the adenine nucleotide (ATP or ADP) binding site (Kabsch and Holmes, 1990). Actin also interacts with several proteins, including other cytoskeletal elements, that influence the polymerized state of actin (Bamburg and Bernstein, 1991). Actin in neurons was first considered to be a general structural protein (Bray, 1977). Subsequent reports have identified a wider role for actin filaments in physiological functions such as secretion, fast axonal transport and in the regulation of growth cone motility (Bamburg and Bernstein, 1991). These functions suggest that actin dynamics are important in the morphological response of neurons to their environment (Pollard and Cooper, 1986).

Factors regulating actin filament assembly

To shape the static and dynamic structure of the neuronal cytoskeleton actin filament assembly and the depolymerization must be regulated. For example, polymerization/depolymerization occurs under specific ionic conditions, and several classes of proteins and non-protein factors strongly

regulate the equilibrium of monomeric and polymeric actin (Bamburg and Bernstein, 1991).

The formation of filamentous actin (F-actin) is initiated by the binding of ATP to the actin monomer (G-actin), which changes the conformation of the protein, resulting in increased affinity of the actin filament for G-actin. The formation and growth of the filament require the initial nucleation of actin monomers into at least an trimer (Lambooy and Korn, 1988). Once nucleation occurs, filaments elongate. The elongation is obviously dependent on the concentration of free actin, so that with lowering of the actin, the elongation reaches a dynamic equilibrium (Pollard and Cooper, 1986). The actin concentration remaining free is called the critical concentration.

Assembly is counteracted by an endogenous process; hydrolysis by the internal ATPase activity of F-actin. The hydrolysis is not involved in the actual formation of the polymer, as the reaction lags behind the polymerization step (Pardee and Spudich, 1982). Following unbinding of the inorganic phosphate, the actin filament destabilizes, resulting in an 'non-equilibrium polymer' (Wegner, 1985). That suggests that a continuous supply of ATP is required to maintain actin in its filamentous form. For example, the turnover of ATP to ADP occurred every 10 seconds in platelets, resulting in estimates that this process consumed up to 50 % of the total cellular ATP production (Daniel et al, 1986). Therefore, depletion of cellular ATP during prolonged whole-cell dialysis predicts a shift of actin filaments into the monomeric form.

In addition, the interaction of calcium or magnesium with actin influences the stability of actin filaments. In vitro assembly requires

complexed magnesium whereas exchange with calcium causes disassembly (Pollard and Cooper, 1986). Although $[Mg^{2+}]_i$ is relatively stable, submembrane calcium fluctuates and can reach high peak concentrations (Augustine and Neher, 1992). Thus calcium transients may become a major factor controlling the actin polymerized state (Forscher, 1989).

Actin can also be modified on the N-terminus as well as by phosphorylation, however the physiological role of this modulation is not understood. An interesting modulation is mediated by Botulinum toxin C2 that ADP-ribosylated actin, resulting in the disorganization of the filamental form (Reuner et al., 1987).

Proteins regulating actin dynamics

At steady state, only 50 % of actin is in the filamentous form, which is less than the 98 % predicted by the available ATP and ionic cellular conditions. This suggests that factors other than the self-assembly properties must regulate assembly (Bray, 1977). This is accomplished with several classes of regulatory proteins (Bamburg and Bernstein, 1991).

Recently there has been an explosive increase in the identification of actin binding proteins (ABP) that somehow regulate the behavior of monomeric or filamentous actin. The function of most ABP's remains to be established (Bamburg and Bernstein, 1991). The function of profilin and gelsolin are better understood and potentially important in the regulation of the NMDA channel. Profilin and gelsolin regulate assembly by buffering the G-actin, and reducing the 'critical concentration' for assembly. The concentration of profilin is approximately equimolar with actin (300 μM),

thus with a K_D of 1 μM, profilin would shift the amount of filamentous actin from 91 % to 49 % (Tobacman et al., 1983). These buffer properties are not necessarily static. Binding of inositol poly- and triphosphates (IP_3), produced by phospholipase C, reduces the actin-binding affinity of profilin or gelsolin (Forscher, 1989; Bamburg and Bernstein, 1991). This could be a general mechanism for the rapid release of actin monomers and subsequent polymerization, that is tightened to the G-protein-dependent IP_3 turnover. Gelsolin also causes severing of actin filaments in higher calcium concentrations, and can act as a stimulator of actin assembly by enhancing nucleation in lower calcium (Forscher, 1989).

The cortical cytoskeleton network

The cellular functions of actin depend on the formation of heteropolymers with other cytoskeletal proteins. The network of cytoskeletal proteins consists mainly of actin and spectrin and underlies the cell membrane (Bretscher, 1991). The function of the cortical cytoskeleton influences the structure and mobility of cells and also is strongly involved in the function and topology of membrane- and membrane associated proteins. The role of the cortical cytoskeleton in neurons could be involved in the partitioning of glycoproteins in the cell membrane, as has been shown for erythrocytes (Edidin et al., 1991). The restricted diffusion "traps the floating icebergs in the sea of lipid", resulting in local enrichment of ion channels or membrane receptors.

Knowledge of structure and function of the spectrin-actin network comes mostly from studies on erythrocytes, where it is responsible for the

elasticity and stability of the cell and the characteristic disc shape (Bennett, 1985). Freeze-fracture and rotary shadow images show that the network has an approximately hexagonal structure, formed by 5-6 spectrin tetramers attached to oligomers (app. 12) of actin (Byres and Branton, 1985; Liu et al., 1987). The spectrin tetramers are formed by head-to-head association of spectrin dimers.

A neuronal isoform of spectrin, fodrin, has a calmodulin binding site that distinguishes it from erythroid forms (Leto et al., 1989). Calmodulin regulates the interaction of spectrin with actin as well as with membrane anchoring proteins (Anderson and Morrow, 1987). Calmodulin binding also enhances the sensitivity of spectrin to protease cleavage (Harris and Morrow, 1990). Thus, these properties of fodrin link the stability of the cortical cytoskeletal network to intracellular calcium.

Attachment of the actin-spectrin network to the membrane is accomplished by ankyrin. Ankyrin has been shown to bind to spectrin (Bennett, 1978), to Na/K ATPases (Koob et al., 1988) and voltage dependent Na channels (Srinivasan et al., 1988). Association in neurons also occurs with a series of yet unidentified proteins (Davis and Bennett, 1986).

The subcellular distribution of ankyrin in neurons provides some perspective on its function. Ankyrin has been detected at the node of Ranvier (Kordeli et al., 1988; Srinivasan, 1988), and in high concentrations at postsynaptic sites (Kordeli et al., 1986). In both cases, it is colocalized with spectrin (Carlin et al., 1983; Koenig and Repasky, 1985), suggesting a concerted action of spectrin/ankyrin. Several forms of ankyrin regulation have been identified. Acylation with palmitic acid leads to a attachment to the

membrane (Staufenbiel and Lazarides, 1986). Cleavage of the amino terminal by calpain I reduces the affinity to the Na/K ATPase (Hall and Bennett, 1987). Ankyrin also serves as a substrate for multiple kinases (Lux et al., 1990); although the functional significance of this phosphorylation is not understood.

Channel-cytoskeleton interaction occur in sensory systems that utilize mechanotransduction, and in the participation of channels to specific neuronal compartments such as synapses, or to the node of Ranvier. Examples and mechanisms for these interactions are discussed in more detail in manuscript III.

MECHANISM OF CHANNEL REGULATION

Modulation of neuronal excitability has been a central issue of neuroscience. As ion channels ultimately determine membrane excitability, the modulation of ion channels has been a major focus of investigators. Although nearly all channels so far studied are modulated by some mechanism, the best studied examples of channel regulation are the L-type calcium channel and the nicotinic receptor. Both channels show some common features to NMDA channel modulation, as identified in this dissertation. Both channels are modulated by either covalent modification such as phosphorylation, and ionic interaction with the divalent Ca^{2+}.

The L-type calcium-channel

The regulation of L-type calcium currents on cardiac myocytes has been studied for 20 years. The contractility of the heart is highly dependent on the calcium influx during the action potential. Stimulation of the autonomic nervous system activates β-adrenergic receptors that enhance calcium currents (Reuter, 1967; Bean, 1985). Subsequent studies have shown that norepinephrine acts via a cAMP-dependent pathway, as the effect of adrenergic agonists could be mimicked by stimulation of G-proteins, by stimulation of cAMP production or by inhibition of phosphodiesterase (Reuter, 1983; Bean, 1985; Trautwein and Hescheler, 1990). A major target of cAMP is PKA, and it has been proposed that the modulation by cAMP of L-type channel is mediated by PKA-dependent phosphorylation (Catterall, 1988).

Although the amino acid sequence of the alpha-1 subunit of skeletal and cardiac L-type channels possess numerous consensus phosphorylation sites, it was initially unclear whether the channel itself or other regulatory proteins were the target of phosphorylation. However experiments in cell-free systems have demonstrated direct effects of phosphorylation on channel activity. For example, incorporation of phosphate into purified channel proteins in lipid vesicles caused increases of up to 1000 % of calcium fluxes (Catterall, 1988). A recent study on cardiac L-type channels also showed that PKA-dependent phosphorylation restored the rapid channel rundown in inside-out patches (Hartzell et al., 1991; Ono and Fozzard, 1992).

Phosphorylation by PKA is not the only form of modulation of L-type calcium channels. Calcium channels undergo virtually complete rundown during whole-cell recording, however complete block of PKA appears to

inhibit only 30 % of the current. Chad and Eckert (1986) have suggested an additional role of calcium in channel rundown in molluscan neurons. Because intracellular perfusion with protease inhibitors reduced rundown, they suggested that a proteolysis-dependent component was involved. The effect of phosphorylation on L-type calcium channels apparently alters the gating kinetics by shifting the voltage necessary for opening towards more positive membrane potentials (Bean, 1989).

Another form of modulation involves a calcium-dependent inactivation of neuronal calcium currents during prolonged depolarizations. This process is calcium specific as the permeable divalent barium has a much reduced effect. Kinetic analysis on guinea-pig myocytes suggested that this effect of calcium is dependent on the accumulation of cytosolic calcium and not from binding of calcium in the channel pore (Yue et al., 1990). The site and mechanism of action of calcium, although proposed to be closely associated to the channel, is not completely clear. In studies on molluscan neurons, an enzymatic mechanism was proposed that implied a rapid and dynamic role of phosphorylation/ dephosphorylation (Eckert and Chad, 1984; Armstrong, 1989). The authors found that the rate of inactivation was accelerated as the neuron was dialyzed with the phosphatase calcineurin, and recovery from inactivation required rephosphorylation. However, the observed kinetics of the phosphorylation process are on a much slower time scale of seconds to minutes, thus not matching the rate of recovery from inactivation.

The nicotinic acetylcholine receptor. Motor neurons of vertebrates use acetylcholine to signal contraction the myocyte. Presynaptic released acetylcholine binds to the AChR, a ligand gated ion channel, leading to an opening of a nonselective cationic pore. The resulting depolarization causes the firing of an action potential. The function of the AChR is therefore similar to AMPA channels that mediate fast excitatory transmission at many excitatory synapses. It is interesting, that a channel that serves essentially as a 'hard-wired' relay station between nerve and muscle apparently undergoes such a variety of modulation.

Large quantities of channel protein can be isolated from *torpedo* electric organs (Raftery et al., 1980) which led to the early cloning of subunits (Noda et al., 1983). Biochemical studies on the isolated AChR receptor have shown that the receptor can be phosphorylated by tyrosine kinase, PKA and PKC at single sites of the β-, γ-, and δ- subunits (Swope et al., 1992). Functional studies demonstrated that purified, reconstituted channels have increased desensitization rates when phosphorylated by PKA or tyrosine kinase (Swope et al., 1992). Studies in intact muscle cells also showed an increase in desensitization by forskolin or cAMP analogs (Middleton et al., 1986; Middleton et al., 1988). However, the role of cAMP-dependent phosphorylation had been challenged by the finding that forskolin alters AChR desensitization by an effect independent of phosphorylation, analogous to the effects of local anesthetics (Wagoner and Pallotta, 1988; White, 1988).

REGULATION OF GLUTAMATE GATED CHANNELS

The AMPA channel

Two recent studies showed that AMPA channel rundown in whole-cell recordings can be prevented by preserved activity of cAMP dependent protein kinase and by inhibition of phosphatases (Greengard et al., 1991; Wang et al., 1991; Rosenmund and Westbrook, unpublished observation). This strongly suggests that channel activity is regulated by phosphorylation of the receptor or a related regulatory protein. The cloning of AMPA channel subunits indicates consensus phosphorylation sites for serine/threonine kinases. The only subtype with a consensus phosphorylation site for PKA is the subunit GluR6 (Egebjerg et al., 1991). However receptor modulation of GluR6 subunits by protein kinases when expressed in cell lines showed somewhat conflicting evidence (Taverna et al., 1992, Soc. Neurosci. Abstr. 18, 651; Raymond et al., 1992, Soc. Neurosci. Abstr. 18, 652; J. Yekel, personal communication). In addition, currents activated by low concentration of S-AMPA, which activates only channel subtypes GluR 1-4 show similar rundown behavior (Rosenmund and Westbrook, unpublished observation). finally, coexpression of GluR1 and GluR3 channel subunits in *Xenopus oocytes* has been reported to activate kainate currents that were modulated by cAMP/PKA-dependent phosphorylation, although GluR1 and GluR3 do not possess PKA consensus phosphorylation sites (Keller et al., 1992). This suggest either that proteins other than the channel molecule are the target of phosphorylation, or that sites other than the consensus phosphorylation

The NMDA channel

NMDA channels are regulated by several extra- and intracellular molecules including calcium, magnesium, protons, polyamines, redox agents, steroids, nitric oxide, and arachidonic acid (MacDermott et al., 1986; Nowak et al., 1984; Mayer et al, 1984; Johnson and Ascher, 1990; Tang et al., 1990; Vyklickly et al, 1990; Lerma, 1992; Wu et al., 1991; Manzoni et al., 1992; Lei et al., 1992; Miller et al., 1992). The physiological relevance of some of these agents, in particular for intracellular modulation, remains to be established.

Regulation of the NMDA channel by phosphorylation has been proposed because rundown of channel activity in whole-cell recordings can be prevented by an ATP regenerating system (MacDonald et al., 1989). Chen and Huang (1991; 1992) also proposed that activation of PKC by μ opioids enhances NMDA channel activity by decreasing the voltage-dependent magnesium block of the channel. Other investigators also reported regulation of NMDA channels by PKC. NMDA channel expressed in oocytes were potentiated by PKC (Kelso et al., 1992), and had inhibitory action in cultured hippocampal neurons (Markram and Segal, 1992). In addition, PKC activator phorbol esters can have nonspecific effects on channels (Hockberger et al., 1989).

The regulation of the NMDA channel by calcium is controversial. Initial experiments proposed an inhibition of NMDA currents by intracellular calcium (Mayer et al., 1987). However other studies seemed to contradict this result (Clark et al., 1990; MacDonald et al., 1989).

Summary

The goal of this dissertation was to characterize the effects of calcium and ATP on two features of NMDA channel activity in whole-cell recording: inactivation and rundown. Three sets of experiments were performed as presented in the following manuscripts.

In manuscript I, the calcium-dependency of NMDA channel inactivation in long pulses of agonist was examined. Whole-cell and cell-attached recordings on cultured hippocampal neurons were employed. Calcium in the cell interior and exterior was manipulated and the kinetics of inactivation and recovery were examined.

Manuscript II examined the rundown phenomenon of NMDA channel activity during prolonged whole-cell recordings. Single cells were internally dialyzed or perfused to determine the cytosolic factors involved. Rundown was $[Ca]_i$ - and ATP -dependent, but apparently did not involve phosphorylation. The change of channel gating underlying rundown was measured.

Manuscript III examined the involvement of actin cytoskeleton in channel rundown. Inside-out and whole-cell recordings were used. The calcium- and ATP-dependent rundown described in manuscript II was found to be due to effects on actin filaments. However, calcium dependent inactivation did not appear to involve actin depolymerization, suggesting an additional effect of calcium. A model integrating the experimental findings is presented in the manuscript III, figure 5 and in the discussion.

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Inactivation of NMDA channels in cultured hippocampal neurons by intracellular calcium

Abbreviated title: Ca^{2+}-dependent inactivation of NMDA channels

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SUMMARY

Calcium-dependent inactivation of N-methyl-D-aspartate (NMDA) channels was examined on cultured rat hippocampal neurons using whole-cell voltage clamp and cell-attached single channel recording. An ATP regeneration solution was included in the patch pipette to retard current 'rundown'. In normal $[Ca^{2+}]o$ (1-2 mM) and 10 μM glycine, macroscopic currents evoked by 15 second applications of NMDA (10 μM) inactivated slowly following an initial peak. At -50 mV in cells buffered to $[Ca^{2+}]_i < 10^{-8}$ M with 10 mM EGTA, the inactivation time constant (t_{inact}) was ≈5 sec. Inactivation did not occur at membrane potentials of +40 mV and was absent at $[Ca^{2+}]o \leq 0.2$ mM, suggesting that inactivation resulted from transmembrane calcium influx. The percent inactivation and t_{inact} were dependent on $[Ca^{2+}]o$. The t_{inact} was also longer with BAPTA in the whole-cell pipette compared to EGTA, suggesting that t_{inact} reflects primarily the rate of accumulation of intracellular calcium.

Inactivation was incomplete, reaching a steady state level of 40-50% of the peak current. At steady state, block of open NMDA channels with MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine) completely blocked subsequent responses to NMDA suggesting that 'inactivated' channels can reopen at steady state. Inactivation was fully reversible in the presence of ATP, but was not blocked by inhibiting phosphatases or proteases. In cell-attached patches, transient increases in $[Ca^{2+}]_i$ following cell depolarization also resulted in inactivation of NMDA channels without altering the single channel conductance. This suggests that Ca^{2+}-dependent inactivation occurs in intact cells and can be triggered by calcium entry through nearby voltage-gated calcium

channels, although calcium entry through NMDA channels was more effective. We suggest that $[Ca^{2+}]_i$ transients induce NMDA channel inactivation either by binding to the channel or a nearby regulatory protein to alter channel gating. This mechanism may play a role in down regulation of postsynaptic calcium entry during sustained synaptic activity.

INTRODUCTION

Calcium acting as a second messenger plays a key role in neuronal function. As a result, intracellular calcium concentrations are tightly regulated by a series of cellular buffers and pumps (Blaustein, 1988; Carafoli, 1987). Thus in most cases the effects of intracellular calcium transients, either from transmembrane influx or via release from intracellular stores, are relatively localized near the source of calcium entry or release. For example, Ca^{2+} transients in 'domains' near presynaptic release sites provide the brief, high concentrations of calcium necessary for transmitter release (e.g. Smith and Augustine, 1988; Llinas et al., 1992). Focal increases in calcium in dendritic spines also occur following synaptic excitation (Müller and Connor, 1991). This compartmentalization has important functional consequences. For example, the induction of long-term potentiation (LTP) in the CA1 region of the hippocampus is triggered by postsynaptic calcium influx through N-methyl-D-aspartate (NMDA), whereas depolarization and opening of voltage-dependent calcium channels is not as effective (Nicoll et al., 1988).

One target of intracellular calcium is voltage-dependent and ligand-gated ion channels (e.g. Scobon-Mulieri and Parsons, 1977; Inoue et al., 1986; Behrends et al., 1988; Chad and Eckert, 1984; Marchenko, 1991). Calcium increases the activity of Ca^{2+}-dependent K^+ or Cl^- channels (Marty, 1989), but decreases the activity of voltage dependent calcium channels (Chad, 1989). Calcium can bind directly to ion channels or act via Ca^{2+}-dependent enzymes (Marty, 1989; Chad and Eckert, 1986; Armstrong et al., 1991). Ca^{2+}-dependent inactivation of calcium channels appears to involve the action of a phosphatase, but other calcium-

dependent processes may also contribute (Chad and Eckert, 1986; Belles et al., 1988; Korn and Horn, 1990). For calcium-permeable channels, inactivation could limit transmembrane calcium flux during prolonged depolarization, and thus could be an important protective factor to minimize Ca^{2+}-induced neuronal death (e.g. Choi, 1988; Lynch and Seubert, 1989).

The *N*-methyl-D-aspartate (NMDA) receptor is subject to modulation by a number of extracellular agents including Mg^{2+}, glycine, Zn^{2+}, and protons, (for review, Mayer and Westbrook, 1987; Collingridge and Lester, 1989), but intracellular regulation is much less clear. Several experiments have suggested that calcium can lead to inactivation (or desensitization) of the NMDA channel, although both intracellular (Mayer and Westbrook, 1985; Mayer et al., 1987) and extracellular mechanisms have been proposed (Zorumski et al., 1989; Clark et al., 1990).

We used whole-cell voltage clamp and cell-attached patch recording to examine calcium-dependent inactivation of NMDA receptors in cultured hippocampal neurons. Calcium influx through NMDA channels resulted in slow inactivation that reached a maximum of 50% of the initial peak current. The rate of inactivation appeared to reflect the rate of intracellular calcium accumulation rather than the kinetics of the Ca^{2+}-dependent process, and did not appear to involve Ca^{2+}-dependent proteases or phosphatases. We suggest that calcium binds either to the NMDA channel or a nearby regulatory protein to regulate the kinetics of the NMDA channel.

METHODS

Cell cultures. Cultures of hippocampal neurons were prepared as previously described (Legendre and Westbrook, 1990). Hippocampi from postnatal day 1 Sprague Dawley rat pups were incubated in a low calcium saline with papain (5-20 U/ml, Worthington Biochemicals) for 1 hour. The papain was inactivated in bovine serum albumin and trypsin inhibitor, the tissue was mechanically dissociated and plated onto a confluent layer of hippocampal astrocytes. The culture medium contained MEM, 0.6% glucose, 5% heat inactivated horse serum (Hyclone) and a supplement including 200 μg/ml transferrin, 200 μM putrescine, 60 nM sodium selenite, 40 nM progesterone, 40 ng/ml corticosterone, 20 ng/ml triiodothyronine, and 10 μg/ml insulin. Cultures were treated one day after plating with a mixture of 5'-fluoro-2-deoxyuridine and uridine (15 and 35 μg/ml, respectively) to suppress overgrowth of background cells; half-changes of medium were done twice weekly.

Whole-cell recording and drug delivery. Whole-cell voltage clamp recordings were performed on neurons after 1-2 weeks in culture. The extracellular solution contained (mM): Na^+ 162; K^+ 2.4; Ca^{2+} 1.3 Mg^{2+} 0; Cl^- 167; Hepes 10; glucose 10, pH adjusted to 7.3. Tetrodotoxin (0.5 μM), strychnine (2 μM) and picrotoxin (50 μM) were added to block spontaneous electrical activity and glycine/GABA channels, respectively. Glycine (10 μM) was also added to facilitate activation of NMDA-evoked currents. In some experiments, $[Ca^{2+}]o$ was varied between nominally free to 50 mM; sodium was adjusted to keep the osmolarity at 325 mOsm. Patch pipettes for whole-cell recording were pulled from borosilicate glass (TWF 150, WPI), coated with Sylgard and fire-polished. DC resistances

were 2-10 MΩ. Pipette solutions contained (mM): CsCl 95; EGTA 10; HEPES, 10; and an ATP 'regeneration' solution (Forscher and Oxford, 1985; MacDonald et al., 1989) including NaATP 4, $MgCl_2$ 4 creatine phosphokinase 50 U/ml and disodium phosphocreatine 20 mM. Patch solutions were prepared daily from frozen stocks and kept on ice until use. The pH was adjusted to 7.2 with CsOH; osmolarity was adjusted to 295 mosM. In some experiments, intracellular calcium was increased using Ca/EGTA mixtures; the pipette calcium concentration was calculated assuming a EGTA dissociation constant of 10^{-7} M. The chamber was continuously perfused (1-2 ml/min) at room temperature (≈20 C.). Whole-cell currents were recorded using an Axopatch 1B (Axon Instruments) with the current filtered at 10 kHz. Membrane current records were monitored on a chart recorder and stored on either on computer or videotape (VR10, Instrutech Corp.). For display purposes, currents were filtered at 30-100 Hz.

NMDA (1 - 300 μM) was dissolved in the extracellular solution and applied via an array of flow pipes (400 μm I.D.) positioned within 100-200 μm of the cell. Each flow pipe was controlled by solenoid values; solutions were changed by simultaneously closing one valve and opening another. The solution exchange time constant was ≤ 20 ms as measured by change in membrane current evoked by kainic acid in two concentrations of sodium (Vyklicky et al., 1990). Ultrapure calcium and sodium salts (Aldrich) were used in the flow pipes for drug delivery. In experiments with F^-, CsF (50 mM) was substituted isotonically for CsCl in the patch solution. Stock solutions of calmidazolium (10 mM in DMSO, Calbiochem) and okadaic acid (100 μg/ml in DMF, Calbiochem) were diluted into the pipette solution before each experiment. Fifteen minutes were allowed after the start of whole-cell recording for the substance to diffuse

into the cell; this is more than sufficient to allow exchange of these low molecular weight compounds (Pusch and Neher, 1988; Oliva et al., 1988). The access resistance was regularly monitored with small voltage steps. Excitatory amino acids were obtained from Cambridge Research Biochemicals or Tocris Neuramin; other chemicals were obtained from Sigma.

In some experiments, voltage jumps were used to evoke voltage-dependent calcium currents. In these experiments extracellular calcium was increased to 5 mM during the voltage jump. Jump protocols and membrane currents were recording on an IBM-PC using pClamp software (v 5.5). No leak subtraction was used.

Single channel recording . Cell-attached recording was made using relatively large patch electrodes (1-2 MΩ) containing Ca^{2+}-free extracellular solution buffered with 10 mM EGTA. L-glutamate (1 μM) and glycine (10 μM) were included in the patch pipette. Single channel currents were stored on a digital data recorder (VR10, Instrutech), then replayed, filtered at 2 kHz (8-pole Bessel, Frequency Devices), digitized at 10 kHz and analyzed on an IBM AT clone using Pclamp software (v. 5.5). Single channel conductance was checked for each patch using the observed reversal potential. Software developed by John Clements (AXOGRAPH, Axon Instruments) was used to plot cumulative point-per-point amplitude histograms and obtain estimates of channel opening probability (np) where n is the number of channels and p is the probability of opening for a single channel.

Results are presented as mean values ± standard deviation. Statistical comparisons were made using the Student's t test.

RESULTS

Whole-cell recordings were performed on hippocampal neurons after 7-14 days in culture. When neurons were clamped near the resting potential in normal extracellular calcium ($[Ca^{2+}]o$ = 1-2 mM); NMDA-evoked inward currents in Mg^{2+}-free solutions showed Ca^{2+}-dependent inactivation in all neurons tested. In order to limit rundown of the NMDA responses during long recordings, an ATP 'regeneration' solution (Forscher and Oxford, 1985; MacDonald et al., 1989) was added to the intracellular solution. This allowed stable recordings for periods up to 30 minutes. In most experiments, 15 sec drug applications were delivered every 100 seconds to reduce cumulative effects of repeated applications.

Slow voltage-dependent inactivation by Ca^{2+}

Both glycine-dependent and glycine-independent desensitization of the NMDA receptor have been observed that are not calcium-dependent (Mayer et al., 1989; Sather et al., 1990). In order to examine Ca^{2+}-dependent inactivation in isolation, neurons were voltage clamped at -50 mV in the presence of low concentrations of NMDA (1-10 μM) and high concentrations of glycine (10 μM). Under these conditions, there was little apparent desensitization/inactivation of the NMDA-evoked inward currents in low $[Ca^{2+}]o$. (Fig. 1A). However when $[Ca^{2+}]o$ was raised to 1.3 mM, a slow current relaxation appeared that approached a steady state level within 10-15 sec. We have called this relaxation 'inactivation' to avoid confusion with other forms of NMDA receptor desensitization. When the membrane potential was shifted to +40 mV, the Ca^{2+}-dependent relaxation was markedly reduced (Fig 1A).

A small residual relaxation was often observed even at positive membrane potentials. This relaxation was unaffected by changes in $[Ca^{2+}]o$, was much slower than the Ca^{2+}-dependent inactivation and was not further analyzed. At a holding potential of -50 mV, there was also a small decrease in the peak current amplitude in 1.3 mM $[Ca^{2+}]_o$ (13 ± 4 %, n=14) compared to 0.2 mM $[Ca^{2+}]_o$. This is consistent with a reduction in the single channel conductance of the NMDA channels as $[Ca^{2+}]_o$ increases (Jahr and Stevens, 1987; Ascher and Nowak, 1988].

We examined several simple possible explanations for Ca^{2+}-dependent inactivation including changes in ion gradients, activation of secondary Ca^{2+}-dependent conductances or a Ca^{2+}-induced change in agonist affinity. However, the reversal potential was unaffected as shown by a 130 ms ramp (1 mV/ms) obtained at the peak and end of a 15 second NMDA application (Figure 1B). The reversal potential was 4.1 ± 2.7 mV at the peak and 4.3 ± 2.9 mV (n=7) after the relaxation reached steady-state. Of note, the slope conductance was reduced over the entire voltage range suggesting that recovery from calcium dependent inactivation was slower than the duration of the ramp (see also Figure 2). Substitution of the impermeant anions gluconate or methylsulfonate for chloride in the intracellular solution also did not affect the degree of Ca^{2+}-dependent inactivation. The inactivation was 47.8 ± 5.9 % (n=4) with gluconate-containing pipettes and 45.2 ± 5.3 % (n=5) with methylsulfonate-containing pipettes, compared to 43.7 ± 6.4 % with chloride-containing pipettes (n=20, see Fig. 3). Thus activation of a inward Ca^{2+}-activated chloride current is not responsible for the Ca^{2+}-dependent relaxation under our conditions.

To test whether Ca^{2+}-dependent inactivation was a result of reduced ligand affinity, we measured the relaxation using a supramaximal concentration

of NMDA (300 μM). At this concentration, a small relaxation (15.0 ± 3.3%, n=10) was seen in the absence of $[Ca^{2+}]o$, presumably reflecting an additional form of receptor desensitization (see e.g. Mayer et al., 1989; Sather et al., 1990). However in 1.3 mM $[Ca^{2+}]o$, the relaxation reached 41.6 ± 11.8 %, (n=10) after a NMDA application of 3.5 sec. This was slightly less than the steady state inactivation, however longer drug applications at high agonist concentrations resulted in irreversible channel rundown (see MacDonald et al., 1989). Thus inactivation is not due to a decrease in agonist affinity.

Ca^{2+}-dependent inactivation was not complete even during prolonged agonist applications, usually approaching a maximum inhibition of 45-50%. This suggested the possibility that 45-50% of the channels are completely inactived by calcium, and the remainder are calcium-*insensitive*.. As MK-801 blocks open NMDA channels but exits extremely slowly from the channel (Huettner and Bean, 1988), we used this property to test for separate channel populations. MK-801 rapidly blocked the steady state NMDA current (Figure 1C, left panel), however no recovery was seen to a test NMDA pulse 1 minute following the end of the NMDA+MK-801 application (trace b, Figure 1C). For 4 neurons the block at 1 minute following NMDA + MK-801 was 97 ± 2 %. In the absence of MK-801, recovery of the NMDA current was complete within 1 minute (not shown, see also Figure 2). This suggests that channels inactivated by calcium reopen during the 40 sec exposure to NMDA + MK-801, and that most (if not all) of the current is carried by channels that undergo Ca^{2+}-dependent inactivation.

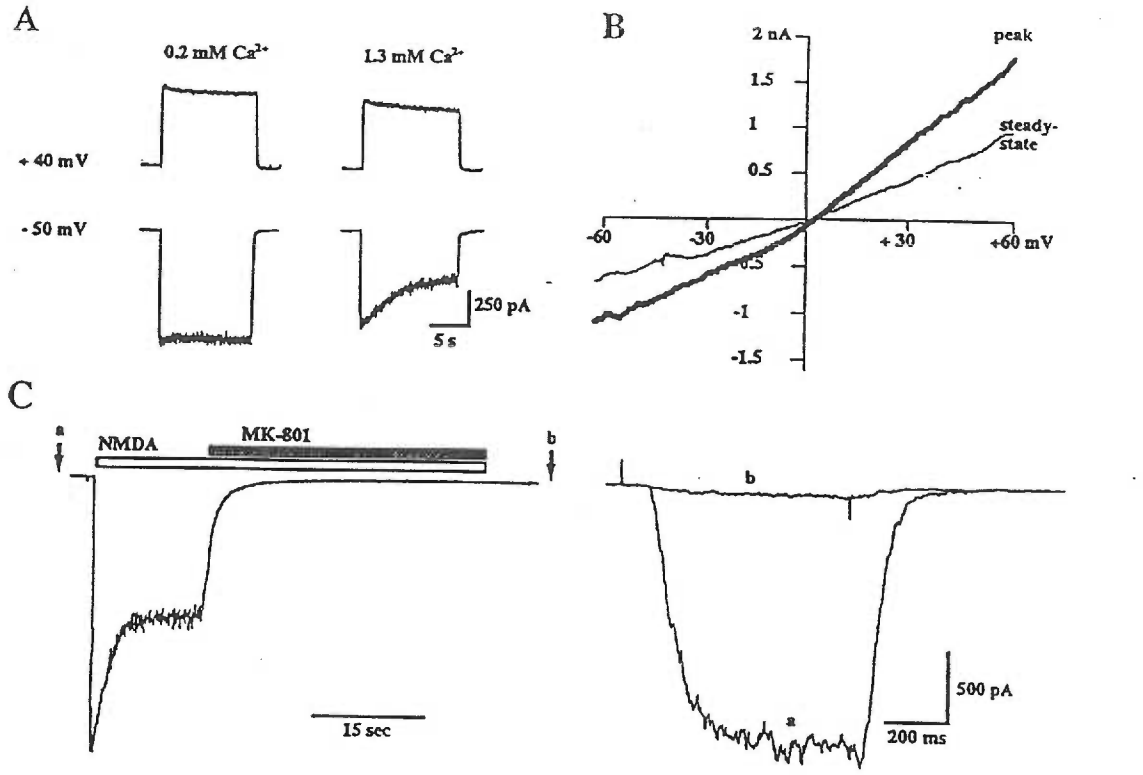

Figure 1. Characteristics of calcium-dependent inactivation of NMDA currents.

A. Whole cell currents were evoked by flow pipe application of NMDA (10 μM, 15 sec) in either low external calcium (0.2 mM, left traces) or normal calcium (1.3 mM, right traces). The current showed no macroscopic desensitization in low calcium at a holding potential of -50 mV, but in 1.3 mM calcium the current inactivated slowly following an initial peak, reaching a steady state level of 47% inactivation for this neuron. The small relaxations seen at +40 mV was calcium-independent.

B. Calcium-dependent inactivation was not due to a change in the ionic driving force. A 130 ms voltage ramp from -70 mV to +60 mV was applied 500 ms after the beginning of the response (solid line), and after inactivation reached steady state at 14.5 s (thin line). The reversal potential was unchanged. Currents are leak subtracted.

C. Inactivation was incomplete but involved the entire population of NMDA channels. NMDA (10 μM) was applied to a neuron at -50 mV. After steady state of inactivation was reached, the current was irreversibly blocked by a 40 sec application of MK-801(10 μM) in the continuous presence of NMDA (left panel). A subsequent test pulses of NMDA (500 msec, 0.2 mM $[Ca^{2+}]o$) 1 minute after removal of NMDA +MK-801 showed no recovery (trace b, right panel), suggesting that all channels opened at some time during the steady state inactivation period.

The onset and recovery from inactivation were examined by a 15 second exposure to 1.3 mM $[Ca^{2+}]_o$ during a prolonged application of NMDA in low $[Ca^{2+}]_o$. As shown in Figure 2A, the onset consisted of a rapid reduction in current resulting from the change in channel conductance followed by a slow relaxation as observed during single applications of NMDA in normal $[Ca^{2+}]_o$. Following return to low $[Ca^{2+}]_o$ solutions, there was complete recovery of the current with a half-recovery time of 22.6 ± 3.7 sec (n=13). The slow recovery did not appear to result from trapping of Ca^{2+} ions in the inactivated channel, because full recovery occurred even in the absence of NMDA(Fig. 2B). In addition the rate of recovery was not increased during continuous exposure to NMDA. This contrasts with the behavior of slow open channel blockers such as phencyclidine, ketamine and MK-801 (Mayer et al., 1988; Huettner and Bean, 1988; MacDonald et al., 1991).

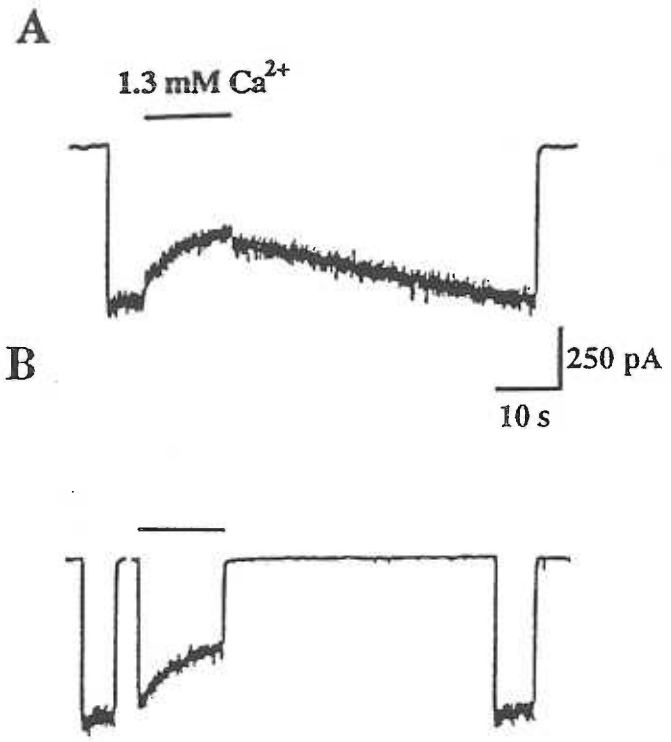

Figure 2. Inactivation was reversible on removal of extracellular calcium.
A. During a long application of NMDA (10 μM, 70 sec), the $[Ca^{2+}]o$ was increased from 0.2 to 1.3 mM for 15 seconds. This was accompanied by an instantaneous reduction in current (due to the reduced single channel conductance) followed by slow Ca^{2+}-dependent inactivation. On return to the low calcium solution recovery was complete. The recovery followed a non-exponential time course with a half recovery time of ≈ 20 sec.
B. Channel activation did not accelerate the recovery rate (compare A with B), suggesting that closed channel block was not responsible for inactivation. NMDA (10μM) was applied in 0.2 Ca^{2+} before, and 50 seconds after, a 15 sec application of NMDA in 1.3 mM Ca^{2+}. As in A, recovery was complete.

Dependence on [Ca]o

Both the maximal inactivation and the rate of inactivation were dependent on the extracellular concentration of calcium. During applications of 10 μM NMDA there was little or no appreciable inactivation during a 15 second application in the absence of added $[Ca^{2+}]o$, however the addition of 0.2, 0.6 and 1.3 mM $[Ca^{2+}]o$ produced a dose-dependent increase in inactivation (Fig 3A). Inactivation was 9.6 ± 11 % at 0.2 $[Ca^{2+}]o$ (n=14), 29.2 ± 2.4 % at 0.6 mM $[Ca^{2+}]o$ (n=6) and 43.7 ± 6.4 % at 1.3 mM $[Ca^{2+}]o$ (n=20). There was no further increase in inactivation when the $[Ca^{2+}]o$ was increased to 10 mM (Fig. 3B). In some neurons, there appeared to be a slight delay in the onset of inactivation at low concentrations of $[Ca^{2+}]o$, as is apparent in Figure 3A. However, the time constant of inactivation (t_{inact}) at 1.3 mM $[Ca^{2+}]o$ was generally well fitted with a single exponential of 4.7 ± 0.6 sec (n=11, Fig. 3C). The t_{inact} decreased as $[Ca^{2+}]o$ increased, i.e. the rate of inactivation was accelerated. Although the degree of inactivation was maximal at 1.3 mM $[Ca^{2+}]o$, t_{inact} continued to decrease, reaching 2.5 ± 0.7 sec (n=8) and 1.2 ± 0.3 sec (n=7) at 10 and 50 mM $[Ca^{2+}]o$, respectively (Fig. 3D). Increasing the concentration of NMDA to 100 μM resulted in a left shift of the calcium concentration-response curve. Inactivation was 28.5 ± 2.6 % (n=4) at 0.2 mM $[Ca^{2+}]o$, , 41.5 ± 2.8 % (n=5) at 0.6 mM $[Ca^{2+}]o$ and 46.6 ± 3.0 % (n=5) at 1.3 mM $[Ca^{2+}]o$.

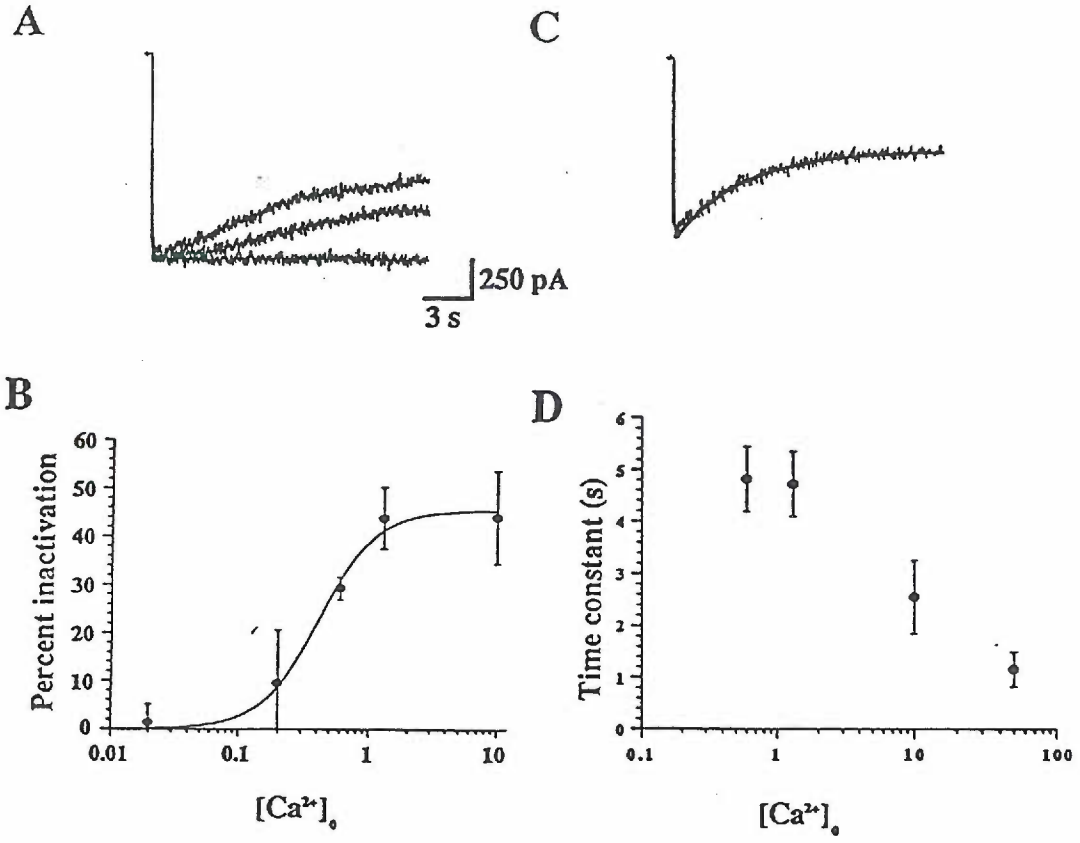

Figure 3. Maximal inactivation and the inactivation rate were dependent on the extracellular calcium concentration.

A. The current evoked by NMDA (10 μM) in 0.2, 0.6 and 1.3 mM $[Ca^{2+}]o$ were normalized to the initial peak and superimposed. The inactivation progressively increased from a 27% reduction in 0.6 mM to 41% in 1.3 mM $[Ca^{2+}]o$ for this neuron.

B. The percent inactivation is shown as a function of extracellular calcium. The nominally calcium-free solution had an estimated calcium concentration of 20 μM. Inactivation was measured at the end of a 15 second application of NMDA (10 μM). Each point is the average of 6 to 20 neurons. Intracellular buffer was 10 mM EGTA with no added calcium. The data were fitted with the logistic equation, $I = I_{max} * 1/(1+(IC_{50}/[Ca^{2+}]_o)^n$ where I=% inactivation; I_{max}=maximal inactivation (45.2%). The half maximal inactivation (IC_{50}) was 411 μM with a slope factor (n) of 1.97.

C. Inactivation was reasonably well fitted by a single exponential with a time constant of ≈ 4 sec in 1.3 mM $[Ca^{2+}]o$ (10 mM EGTA buffering).

D. Increases in $[Ca^{2+}]o$ above 1.3 mM accelerated the inactivation time constant without altering the maximal inactivation. Each point is the average of 6 to 11 neurons.

Dependence on $[Ca^{2+}]i$

NMDA channels are calcium permeable thus Ca^{2+}-dependent inactivation could result from accumulation of intracellular calcium. Although the experiments above were performed using intracellular buffering with 10 mM EGTA, the increased inactivation at high concentrations of agonist suggested that the degree and rate of inactivation were dependent on the buffer capacity in the cell. As BAPTA is a much more rapid and selective Ca^{2+} buffer, we examined inactivation during dialysis with 15 mM BAPTA. As shown in Figure 4A, there was no apparent inactivation during a 15 sec application of 10 μM NMDA at 1.3 mM $[Ca^{2+}]o$,. However the effect of BAPTA could be overcome by raising the $[Ca^{2+}]o$ to 10 mM. For the neuron shown in Figure 4A, the inactivation was 51.6 % with a time constant of 3.8 sec; similar results were obtained on 6 neurons. This also suggests that H^+ or Mg^{2+} transients, resulting from exchange with Ca^{2+} bound to EGTA, do not trigger inactivation.

As noted above, t_{inact} was dependent on $[Ca^{2+}]o$. This could result from the kinetics of a Ca^{2+} binding site or could simply reflect the rate of accumulation of calcium at an intracellular effector site near the channel. Our results suggest the latter as reducing EGTA from 10 to 0.1 mM produced a marked decrease in t_{inact} (Fig. 4B). The t_{inact} was 1.7 ± 0.1 sec (n=6) at 0.1 mM, which was only slightly slower ($p < .02$) than t_{inact} in the absence of added buffer (1.2 ± 0.2 sec, n=7).These results strongly suggest that an intracellular calcium transient following the opening of NMDA channels is responsible for inactivation. Thus we tested whether inactivation could be occluded by tonic increases in $[Ca^{2+}]i$. Ca^{2+}-dependent inactivation was examined in cells dialyzed with either 1 μM Ca^{2+} (= 10 mM Ca^{2+} +11 mM EGTA) or 1 mM Ca^{2+} (= 10 mM EGTA + 11 mM Ca^{2+}). As

shown in Figure 4C, the % inactivation was significantly less using the high calcium patch solutions compared to control cells dialyzed with 10 mM EGTA with no added calcium. Inactivation was 26.4 ± 5.8 % (n=6, $p < .01$) for 1 μM Ca^{2+} and 18.4 ± 8.9 % (n=5, $p < .01$) for 1 mM Ca^{2+} . This compares to 43.7 ± 6.7 % (n=20) inactivation in control conditions (see Fig. 3B). This is the expected result if high intracellular calcium tonically inactivates NMDA channels. The t_{inact} was also decreased (Fig. 4C) similar to cells with low concentrations of EGTA (Fig. 4C.), indicating a rapid accumulation of free calcium. It was somewhat surprising that inactivation was not completely occluded even with 1 mM Ca^{2+} in the pipette solution i.e. in the virtual absence of a calcium gradient, $[Ca^{2+}]o = 1.3$ mM. Thus the free calcium concentration in the submembrane compartment is likely to be less than in the pipette solution, presumably reflecting continuing clearance of calcium via transmembrane pumps and exchangers.

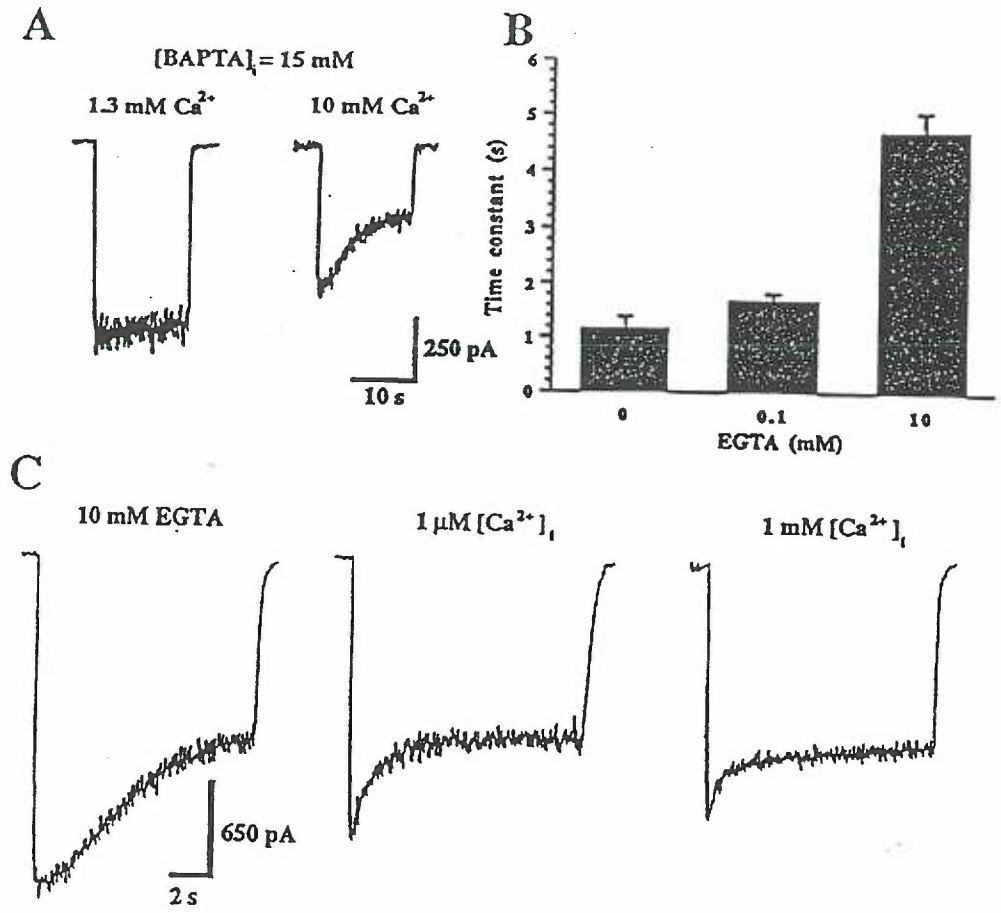

Figure 4. Intracellular calcium buffering also influenced inactivation.
A. Inactivation was significantly reduced using BAPTA compared to EGTA in the whole cell pipette. For this neuron, there was little inactivation in 1.3 mM $[Ca^{2+}]o$ in the presence of 15 mM BAPTA. However, even buffering with 15 mM BAPTA could be overcome with increases in $[Ca^{2+}]o$ to 10 mM, resulting in maximal (≈50%) inactivation.
B. The t_{inact} decreased when EGTA was reduced to 0.1 mM or omitted from the whole cell pipette. $[Ca^{2+}]o$ was 1.3 mM. This suggests that t_{inact} reflects primarily the rate of $[Ca^{2+}]i$ accumulation. Each point is the average of 6-20 cells. Data for 10 mM EGTA are the same as shown in Figure 3D for 1.3 mM $[Ca^{2+}]o$.
C. Ca^{2+}-dependent inactivation was also reduced by increasing the buffered concentration of calcium in the whole cell dialysate to 1 μM (11 EGTA + 10 mM Ca^{2+}) or 1 mM (10 EGTA + 11 mM Ca^{2+}). The control patch solution contained 10 mM EGTA with no added calcium. $[Ca^{2+}]o$ was 1.3 mM, and 5 minutes of whole cell dialysis preceded application of NMDA. With high intracellular calcium, the percent inactivation was markedly reduced and t_{inact} decreased. Examples from three neurons are shown.

Under physiological conditions, several sources may contribute to increases in cytoplasmic calcium including voltage- and ligand-gated channels as well as release of calcium from intracellular stores (e.g. Miller, 1991). We examined whether calcium flux through voltage-gated calcium channels could also induce inactivation of NMDA channels. Increases in cytoplasmic calcium were elicited either by voltage jumps or by prepulses pulses of NMDA in Ca^{2+}-containing medium. The prepulse was followed by a test pulse of NMDA in Ca^{2+}-free medium. As shown in Figure 5A a 240 ms voltage step to +10 mV (5 mM $[Ca^{2+}]o$) produced a sustained inward calcium current. Following 250 msec in calcium-free medium, the NMDA current activated by the test pulse was inhibited by 32.0 ± 9.8 % (n=14). The reduction in the size of the NMDA current could be attributed to transmembrane calcium influx as steps to +70 mV near the Ca^{2+} equilibrium potential had no effect. The inhibition of the test pulse of NMDA was immediate, but then decreased during the pulse, presumably reflecting clearance of calcium from the effector site. Prepulses of NMDA (50 μM, 5 mM Ca^{2+}, 150 ms) in Ca^{2+}-containing medium produced a similar degree of inhibition (34.0 ± 8.9 %, n=6, Figure 5B). This could not be attributed to Ca^{2+} *-independent* desensitization as there was little macroscopic desensitization following application of 50 μM NMDA with $[Ca^{2+}]o = 0$.

Although NMDA channels are calcium permeable, the calcium flux represents only a fraction of the total current (Mayer and Westbrook, 1987) compared to the high selectivity of the calcium channel (Lee and Tsien, 1984). However charge transfer was 114 ± 35 pC compared to 249 ± 58 pC for the prepulse of NMDA. If calcium entry through Ca^{2+} channels was equally effective in producing inactivation of the NMDA channel, this would require that 45% of

the NMDA current was carried by calcium ions. This is much in excess of the estimates from the GHK current equation (Mayer and Westbrook, 1987), and suggests that the Ca^{2+} influx through NMDA channels is more effective.

The recovery from inhibition following a voltage step to +10 mV is shown in Figure 5C. Recovery had a time constant of 12.7 ± 6.3 sec (n=3) with a final recovery of 88 ± 11 % (n=4). This was more rapid than recovery from Ca^{2+}-dependent inactivation produced by long application of NMDA (see Figure 2). These observations suggests that calcium entry, not receptor activation, is responsible for inactivation.

Divalent selectivity and role of Ca^{2+}-dependent enzymes

In addition to Ca^{2+}, several other divalent cations including Ba^{2+}, Sr^{2+} and Mn^{2+} can permeate the NMDA channel (Mayer and Westbrook, 1987; Ascher and Nowak, 1988). However most Ca^{2+}/calmodulin dependent enzymes show marked selectivity for Ca^{2+} compared to other divalent cations (Chao et al., 1984). For example Ca^{2+}-dependent phosphatase that dephosphorylates voltage-dependent calcium currents cannot be activated by barium (Chad and Eckert, 1986). However in our experiments, Ba^{2+} and Sr^{2+} were partially effective in mimicking Ca^{2+}-dependent inactivation of NMDA channels. The results for one neuron are shown in Figure 6; the peak current varied according to the predicted slope conductances for these divalents (Mayer and Westbrook, 1987). In the presence of 1.3 mM [Ba^{2+}] or [Sr^{2+}], inactivation was 28.4 ± 1.4 % (n=7) and 27.2 ± 2.2 % (n=8), respectively. This was significantly less than that due to equimolar Ca^{2+} on the same neurons (41.4 ± 4.5 %, n=8). For the neuron shown in Figure 6, t_{inact} for Ca^{2+} was 3.9 sec compared to 2.8 and 1.3 sec for Ba^{2+} and Sr^{2+}.

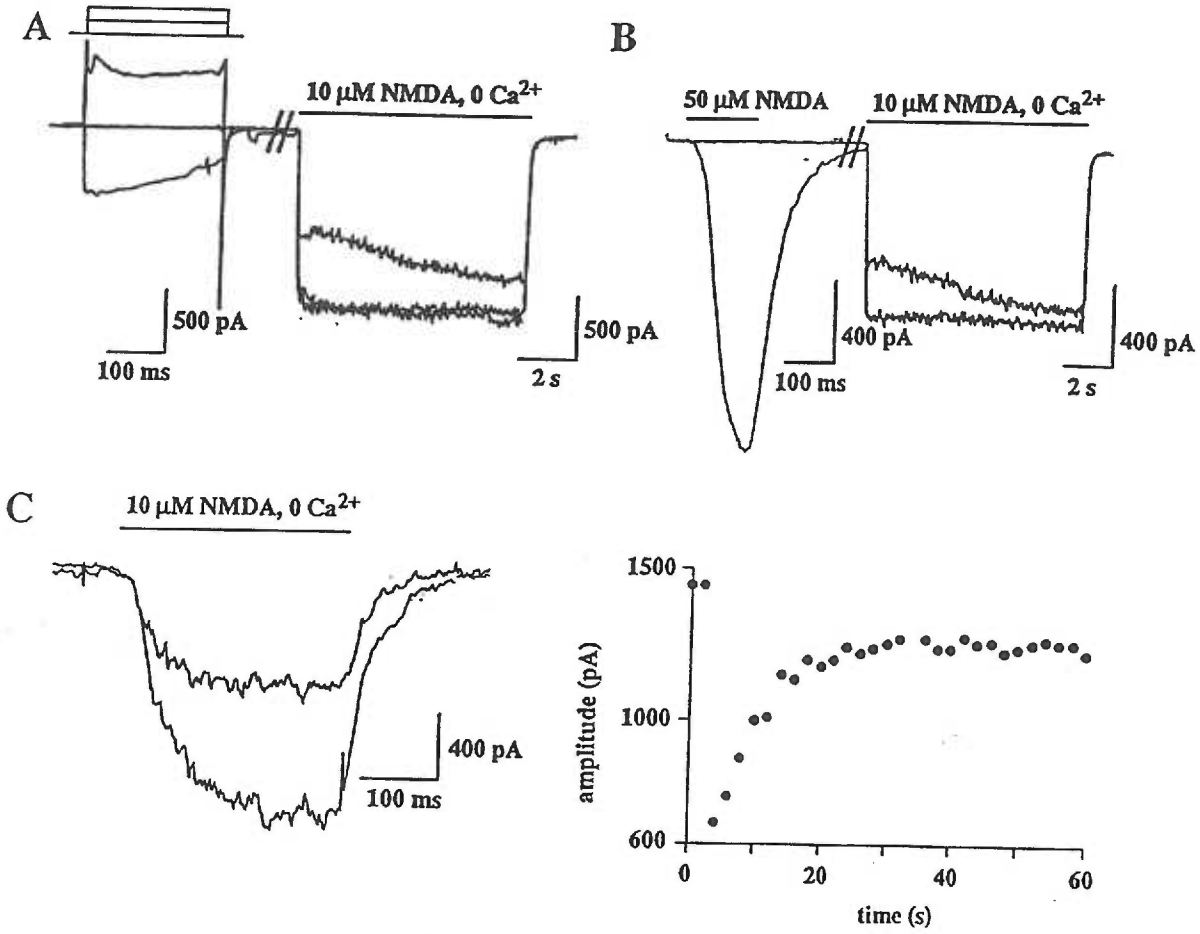

Figure 5. The onset of inactivation required less than 500 ms
A. Voltage jump from a holding potential of -50 mV (240 ms, Vc +10, $[Ca^{2+}]o$ = 5 mM) resulted in an inward calcium current. Following a delay of 250 ms in Ca^{2+}-free medium, the NMDA current (10 μM, $[Ca^{2+}]o$ = 0) was instantaneously reduced by 40% in this neuron . However a voltage jump to the calcium equilibrium potential (+70 mV) evoked an outward current, but had no effect on the NMDA current amplitude. The NMDA current following the jump to +70 mV is superimposed on the current in the absence of a voltage prepulse. The whole cell pipette contained 1 mM EGTA with no added calcium and 100 μM leupeptin.
B. A brief pulse of NMDA (50 μM) in Ca^{2+}-containing medium (5 mM) also caused a rapid inhibition of the test NMDA pulse. Same conditions as A.
C. Recovery from inactivation was monitored by 300 ms NMDA pulses in Ca^{2+}-free medium. Test pulses immediately preceding (a), and following (b) a 250 ms voltage jump to +10 mV (see A) are shown in the left panel. Recovery was nearly complete (88%) at 60 sec following the voltage pulse (right panel).

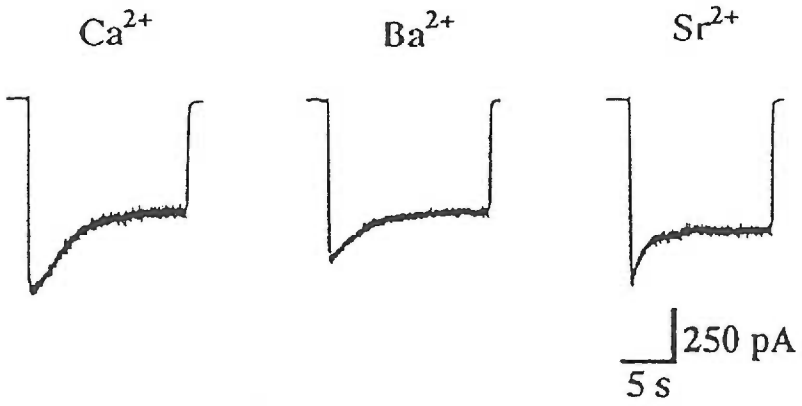

Figure 6. Barium and strontium are less effective than calcium
NMDA (10 μM) was applied in the presence of $[Ca^{2+}]o$, Ba^{2+}, or Sr^{2+} all at 1.3 mM. Responses are from one neuron at a holding potential of -50 mV. The percent inactivation was 41.8%, 29% and 23.8 % respectively. The t_{inact} was decreased for Ba^{2+} and Sr^{2+} compared to Ca^{2+} (see text).

We directly tested whether a protease or phosphatase could be responsible for Ca^{2+}-dependent inactivation. The protease inhibitor leupeptin (100 μM, n=3) had no effect on inactivation when included in the patch pipette. Likewise inactivation was reversible, inconsistent with a proteolytic mechanism. Several phosphatase inhibitors were also introduced in the whole-cell pipette and allowed to diffuse into the cell over 10-15 minutes. Neither the nonselective phosphatase inhibitor Fl^- (50 mM) nor the Type I/2A inhibitor okadaic acid (5 μM) had any effect on the degree or rate of Ca^{2+}-dependent inactivation (n=4). The % inactivation was 44.4 ± 5.8 % at the beginning of recording compared to 43 ± 5.7% following dialysis with okadaic acid. The calmodulin inhibitor calmidazolium (10 μM) also had no effect on inactivation in 4 neurons.

NMDA channel activity in cell-attached patches

Cell-attached recording provides one method to analyze ion channel regulation without disrupting the intracellular milieu. NMDA-activated channels in cell-attached patches have kinetics similar to outside-out patches (Gibb and Colquhoun, 1992). In cell-attached patches from cultured hippocampal neurons, low concentrations of L-glutamate (1 μM) activated inward single channel currents with amplitudes of 3 - 3.5 pA in normal extracellular [Ca^{2+}]. The channels were absent when L-glutamate was omitted from the pipette solution or when 50 μM 2-amino-5-phosphonovalerate (AP5) was added (n=5), consistent with selective activation of NMDA channels. Depolarization of the patch resulted in outward single potassium channel currents, but these were rare at a command potential of 0 mV (the cell resting potential). Although the cell membrane

potential was not controlled, measurement of the reversal potential of the NMDA channels gave a predicted resting potential of -60 to -70 mV.

The goal of these experiments was to assess the effect of increases in cytoplasmic calcium on NMDA channels in the patch. To eliminate calcium flux through channels in the patch, the cell-attached pipette contained 10 mM EGTA. In the absence of $[Ca^{2+}]o$, the single channel current was 4.5 to 5 pA. When the cell was briefly depolarized with 5 sec pulses of either 150 mM KCl + 5 mM Ca^{2+} (or NMDA (100 μM + 5 mM Ca^{2+}) there was an abrupt outward current and disappearance of NMDA channels as the driving force collapsed. Following removal of the KCl solution, the membrane potential was quickly restored as indicated by the return of the single channel current to its basal level (Fig. 6A). However the activity of NMDA channels in the patch was markedly decreased. Similar decreases in activity with applications of KCl or NMDA were not seen when the *pipette* solutions contained 1.3 mM $[Ca^{2+}]$. This suggests that the continuous activity of NMDA channels in the patch is sufficient to induce maximal Ca^{2+}-dependent inactivation, and that depolarization alone cannot account for the decrease in channel activity.

The amplitude histograms immediately before and after KCl are shown in Figure 6B; the probability dropped from 0.64 to 0.26. The time course of the drop in channel activity after KCl application was estimated by calculating the probability in 1 sec epochs. For the patch shown in Figure 6C, the activity returned to baseline levels within 15 seconds. For three patches, the inhibition was 57 ± 8 % which was similar to the maximal inactivation in whole-cell recording.

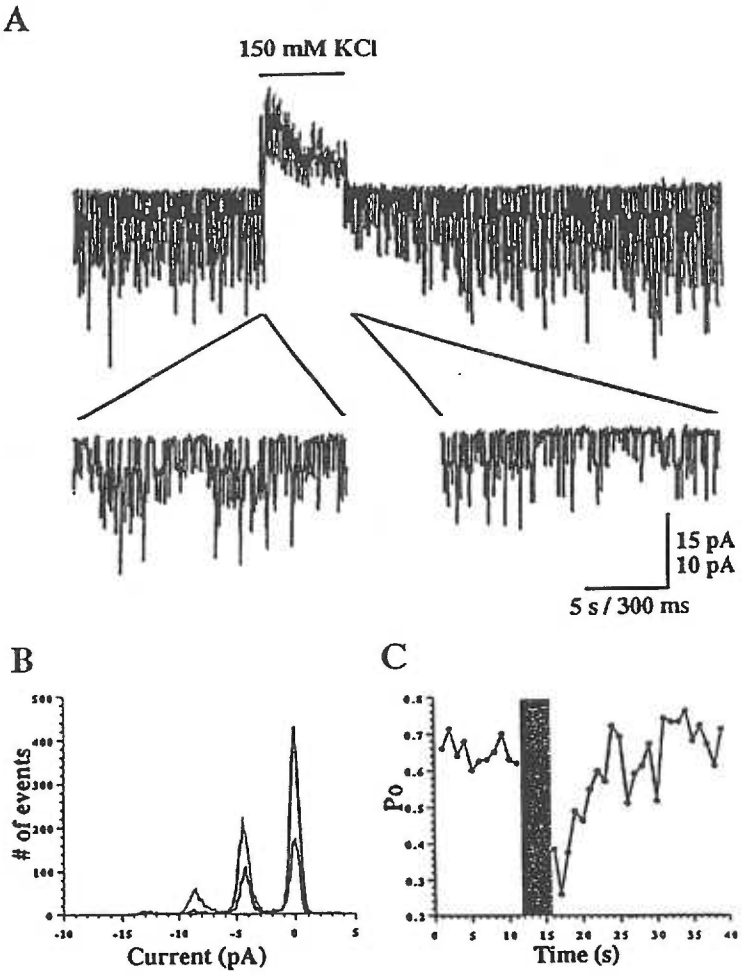

Figure 7. Ca^{2+}-dependent inactivation is present in intact cells
A. In cell-attached recording, L-glutamate (1 μM) activated inward single channel currents of 4.5 pA (Na^+ 165, 0 Ca^{2+}); the patch pipette contained 10 mM EGTA with no added Ca^{2+} to eliminate Ca^{2+} influx through channels in the patch. The patch was clamped at 0 mV. The cell membrane potential was determined by measuring the channel reversal potential, which was usually near -60 mV for stable patches. Cell depolarization (5 sec, 150 mM KCl, 5 mM Ca^{2+}) caused an abrupt outward current. Following removal of the depolarizing stimulus, the membrane potential quickly returned as indicated by the return of the single channel current amplitude to its basal level. However the activity of NMDA channels in the patch was transiently decreased. One second epochs immediately before and after KCl are shown on an expanded time scale.
B. Point by point amplitude histogram of data segment immediately before (thin line) and after KCl (thick line) show the drop in the current peaks at 4.4 and 8.8 pA. The open probability dropped from 0.64 to 0.26.
C. Time course of recovery of NMDA channel activity is plotted in 1 sec epochs. The half-recovery time was 8 sec for this patch. Black bar indicates period of KCl application. Same patch as A,B.

DISCUSSION

Our results demonstrate that intracellular calcium leads to inactivation of the NMDA channel. The rate and degree of inactivation was related to the influx and clearance of intracellular calcium and could be triggered either by opening of NMDA or voltage-dependent calcium channels. Inactivation was unaltered by phosphatase inhibitors suggesting that dephosphorylation of the channel does not underlie Ca^{2+} dependent inactivation.

Ca^{2+}-dependent inactivation results from increases in intracellular calcium

Several distinct mechanisms appear to underlie desensitization of NMDA channels. At submaximal concentrations of glycine, glutamate binding results in an allosteric reduction in glycine affinity with subsequent unbinding of glycine and channel closure (i.e. glycine-dependent "desensitization" (Mayer et al., 1989). However in outside-out membrane patches, prominent glycine-independent desensitization leads to ≥90% loss of the evoked current (Sather et al., 1990; Lester and Jahr, 1992). Profound glycine-independent desensitization also appears in whole cell recording of small or acutely dissociated neurons (Sather et al., 1990; Zilberter et al., 1991). Neither of these phenomena contributed to the Ca^{2+}-dependent inactivation seen in our experiments because glycine was used at saturating concentrations, and macroscopic glycine-independent desensitization was small, representing at most 20% of the peak current at high agonist concentrations. In addition, no macroscopic desensitization was observed with the low agonist concentrations (e.g. 10 μM NMDA) used.

The first indication that calcium might inactivate NMDA channels came from voltage clamp studies of cultured spinal cord neurons (Mayer and Westbrook, 1985). Subsequent experiments using the calcium indicator dye arsenazo III also suggested that increases in intracellular calcium could decrease NMDA responses (Mayer et al., 1987). However recently it has been suggested that the calcium acts at an extracellular site to enhance receptor desensitization (Clark et al., 1990). Increases in extracellular calcium do reduce the conductance of NMDA channels (Mayer and Westbrook, 1987; Jahr and Stevens, 1987; Ascher and Nowak, 1988), but this process is instantaneous and thus clearly distinguishable from the slow inactivation observed in our experiments. Our evidence strongly favors an intracellular site of action for inactivation. Both the degree and rate of inactivation were dependent on the extracellular [Ca^{2+}], but this was highly dependent on the concentration of EGTA in the whole-cell pipette. Lowering the EGTA concentration would be expected to increase the rate of accumulation of intracellular free calcium. Thus the decrease in t_{inact} as the EGTA concentration was decreased (or [Ca^{2+}]o increased) suggests that accumulation of [Ca^{2+}]i determines the inactivation rate. Similar results were seen with BAPTA excluding the possibility that intracellular protons or Mg^{2+} (Johnson and Ascher, 1990) are responsible for inactivation. However even strong buffering with high concentrations of BAPTA could be overcome when the calcium influx was sufficiently large. This may explain the failure of other investigators to see a dependence on intracellular buffering (e.g. Clark et al., 1990)

Calcium-dependent inactivation had several characteristic features. The inactivation was slow and was enhanced at negative holding potentials which

could be explained by calcium influx through open NMDA channels (Mayer et al., 1987). Although we did not determine the calcium sensitivity of the intracellular site, even quite high pipette calcium concentrations did not completely occlude inactivation. This may suggest that the site of calcium action has a relatively low affinity. This would predict a rapid off-rate, thus the slow recovery from inactivation suggests that $[Ca^{2+}]i$ clearance is the rate-limiting step in recovery. Inactivation was maximal at physiological levels of extracellular calcium and also was present in intact cells as measured by cell-attached recording. Thus inactivation may be an important process during normal synaptic transmission. Inactivation was also incomplete, reaching a steady state near 50% of the peak current, however this could not be explained by complete inactivation of a subpopulation of channels, although a small population of Ca^{2+}-insensitive channels cannot be excluded.

Comparison to Ca^{2+}-dependent inactivation of other ion channels

Desensitization or inactivation of several ligand-gated ion channels is calcium-dependent. In the case of calcium permeable channels this could serve as a regulatory mechanism. Although the molecular action of calcium is not known in all cases, several mechanisms appear to be involved. Manthey (1966) first reported increases in desensitization of the muscle AChR by increases in $[Ca^{2+}]o$ which was subsequently attributed to increases in intracellular calcium (Scubon-Mulieri and Parsons, 1977). However, this has not been observed in all preparations (Anwyl and Narahashi, 1980) and potentiation of neuronal AChRs via an extracellular action of Ca^{2+} has also been reported (Vernino et al., 1992). Increases in intracellular calcium can also suppress $GABA_A$ receptors in some

neurons (Inoue et al., 1986; Behrends et al., 1988, but see Shirasaki et al., 1992). Phosphorylation enhances muscle AChR receptor desensitization and it has been proposed that calcium entry through the channels could activate protein kinase C leading to channel phosphorylation (reviewed in Huganir and Greengard, 1990). On the other hand, rundown of GABAA currents in hippocampal neurons is accelerated by intracellular calcium suggesting the a role for dephosphorylation by a Ca^{2+}/CaM dependent phosphatase (Chen et al., 1990).

Perhaps the best studied examples of calcium-dependent inactivation are L-type calcium channels in molluscan neurons (Chad, Eckert and Ewald, 1984). Channel activity in these cells is dependent on cAMP-dependent phosphorylation. Inactivation by intracellular calcium appears to result from Ca^{2+} -dependent dephosphorylation as it is mimicked by perfusion with the Ca^{2+} -dependent phosphatase calcineurin (Chad, 1989; Armstrong, 1989). An additional irreversible component is blocked by leupeptin suggesting an additional component due to a Ca^{2+} -dependent protease (Chad and Eckert, 1986). Ca^{2+} -dependent enzymes have also been suggested as contributing to calcium channel inactivation in GH3 cells and guinea pig myocytes (Belles et all., 1988; Kalman et al., 1988). Although Ca^{2+} -dependent inactivation of the NMDA currents is similar in many of the criteria proposed for inactivation of molluscan calcium channels (Chad, 1989), the mechanisms differ in two important respects. First, Ca^{2+} -dependent inactivation of NMDA currents does not appear to involve phosphatases, and was fully reversible suggesting that proteases are not involved. Secondly, equimolar substitution of Ca^{2+} with Ba^{2+} or Sr^{2+} are partially effective in producing inactivation. Ba^{2+} and Sr^{2+} have larger ionic radii and thus are less effective agonists than Ca in stimulating calmodulin-dependent

processes (Chao et al., 1984). Ba^{2+} and Sr^{2+} did evoke relaxations that were more rapid than Ca^{2+} , perhaps suggesting that buffering and clearance mechanisms are also less efficient in handling divalents other than calcium (see e.g. Ahmed and Connor, 1979) . Thus a mechanism other than dephosphorylation appears to underlie Ca^{2+} -dependent inactivation of the NMDA receptor. However this does not exclude the possibility that the NMDA channel can be phosphorylated as the NR-1 subunit does have a consensus CaM-K II/protein kinase C phosphorylation site (Moriyoshi et al., 1991) and PKC-dependent potentiation of NMDA responses has been reported (Chen and Huang, 1991).

Mechanism and site of action of intracellular calcium

Other than dephosphorylation, calcium could conceivably act by direct binding to the channel, binding to regulatory proteins or by screening negative charges on the membrane. Although our experiments do not directly address these possibilities, they provide constraints on the possible mechanisms and site of action. For example, neither ligand binding nor channel opening were required to elicit Ca^{2+} -dependent inactivation suggesting that the putative binding site is accessible from the cytoplasm, but unlikely to be deep within the channel pore. Likewise, inactivation was not overcome by increasing the agonist concentration, thus a competitive reduction in the affinity of ligand binding is excluded. Recovery also continued in the presence of agonist when extracellular calcium was removed unlike the use-dependence that occurs with closed channel block (e.g. Huettner and Bean, 1988). *Intracellular* Mg^{2+} can bind within the NMDA channels and reduce its conductance , but Ca^{2+} even at 1 mM did not produce this effect (Johnson and Ascher, 1990). In our experiments the I-V

relationship of the inactivated whole-cell current was unchanged from control which excludes a voltage-dependent block by an intracellular divalent cation as the cause of inactivation. The reversal potential of the inactivated current was also unchanged, thus membrane charge screening by $[Ca^{2+}]i$ at concentrations that induce inactivation must be negligible. The calcium-induced reduction in channel activity in cell-attached patches suggests an alteration in channel gating with no apparent effect on single channel conductance. Overall our results are consistent with an allosteric binding site on the channel, but do not exclude an action on a Ca^{2+} -dependent regulatory protein.

Although the site of calcium action does not appear to be within the channel pore, calcium influx through NMDA channels was more effective in the induction of inactivation than influx through voltage-dependent calcium channels. This suggests that the site of calcium action is relatively near the intracellular domain of the receptors. Although the relative distribution of voltage-dependent calcium and NMDA channels is unknown, several observations suggest compartmentalization of intracellular calcium transients following activation of glutamate channels (Malenka et al., 1988; Müller and Connor, 1991). This may involve both channel location or variations in calcium clearance in different compartments (Guthrie et al., 1991). Although an action of calcium near the channel might be expected to have a rapid onset, inactivation was slow during NMDA applications due to the time required for calcium accumulation. Maximal inactivation could occur in ≤500 ms following rapid increases in $[Ca^{2+}]i$ by voltage steps. An interesting possibility is that calcium accumulation leads to progressive changes in channel gating, as has been proposed for calcium-mediated inactivation of voltage-dependent calcium

channels (Yue et al., 1990). This could also be significant during high frequency stimulation of excitatory synapses in the hippocampus.

Role of high energy phosphates

The activity of NMDA channels during whole-cell recording has been reported to require high energy phosphates possibly for direct receptor phosphorylation (MacDonald et al., 1989). We have also noted loss of NMDA current in the absence of ATP ("rundown"), and thus an ATP regeneration solutions was included in our experiments. Under these conditions the NMDA current was stable for recording periods up to 30-60 minutes. However, in the absence of ATP, not only did the peak current decrease, but there was a nearly complete loss of the Ca^{2+} -dependent relaxation (Rosenmund et al, 1991). This could mean that a channel that has 'run down' is no longer subject to Ca^{2+} -dependent inactivation, or that rundown itself is a Ca^{2+} -dependent phenomenon as has been reported for $GABA_A$ channels (Chen et al., 1990), and in our preliminary experiments with NMDA channels (Rosenmund et al., 1991). Thus intracellular Ca^{2+} may play a central role in the regulation of NMDA channels and provide negative feedback control over calcium influx into dendritic spines.

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INSTRUCTIONS TO AUTHORS

The Journal of Neuroscience is the official journal of the Society for Neuroscience. Its purpose is to publish papers on a broad range of topics of general interest to those working on the nervous system.

General Information

Manuscripts must be written in English. Papers should be typed with double spacing throughout, including references, tables, and figure legends; footnotes are not permissible.

Multiple part papers are strongly discouraged. Although it is recognized that this arrangement is sometimes necessary, authors will often be asked to collapse multiple papers into a single manuscript.

Submission of a manuscript to the *Journal* involves the tacit assurance that no similar paper, other than an abstract, has been, or will be, submitted for publication elsewhere; submission also implies thorough understanding of—and concurrence with—the Society's statements on animal treatment and ethics (see below). *Duplicate publication of research results is not acceptable.* Authors must therefore submit five reprints of papers describing any potentially overlapping earlier work (including papers in press). Manuscripts submitted under multiple authorship are reviewed with the understanding that all listed authors concur in the submission and that the final manuscript has been approved by all authors. The copyright, which must be signed by each author, is vested in the Society for Neuroscience.

Five copies of each manuscript (including illustrations) should be prepared. Four copies should be submitted to the Editor-in-Chief; one copy should be sent to the appropriate Section Editor. *Original artwork should be submitted only after provisional acceptance of a manuscript for publication. Manuscripts and artwork will not be returned unless specifically requested by authors.*

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Revised manuscripts and all correspondence should be sent to *both* the Editor-in-Chief and the relevant Section Editor. Revised manuscripts need not be accompanied by a copy of the original version. *Revised manuscripts not returned to the* Journal *within three months will be treated as new submissions.*

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Accepted manuscripts are published with the understanding that the authors will pay the established page charge (currently $40 per published page, excluding fold-outs or color reproductions). The price for all work requiring special printing, including figures in color, will be determined by the publisher (see *Illustra-*

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Manuscripts must include a title page, an abstract, an introductory statement (without heading), a description of the experimental procedures or methods, a description of the results, a discussion of the experimental findings, and, on separate pages, a list of references, figure legends, and tables.

Title page

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The abstract (*not to exceed 300 words*) should state the research objective, procedures, results, and significance of the data. The abstract should be written in complete sentences and in a form acceptable for abstracting services.

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The materials and methods section should be brief but adequate to allow a qualified investigator to repeat the research (see also *Policy Concerning Availability of Materials* below). Reference should be made to published procedures wherever possible; this applies to both the original description and pertinent published modifications.

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These two sections should be as concise as possible. The discussion section should include a brief statement of the principal findings, a discussion of the validity of the observations, a discussion of the findings in light of other published work dealing with the same or closely related subjects, and a statement of the possible significance of the work. Extensive discussion of the literature is discouraged.

References

References should be cited in the text as follows: "The procedure used has been described elsewhere (Green, 1978)," or "Our observations are in agreement with those of Brown and Black (1979) and of White et al. (1980)," or with multiple references, in chronological order: "Earlier reports (Brown and Black, 1979, 1981; White et al., 1980; Smith, 1982, 1984) . . . ." Each listed reference should be cited in text, and each text citation should be listed in the reference section.

In the list of references (to be typed double-spaced) papers should be given in alphabetical order according to the name of the first author. In two-author papers with the same first author, the order is alphabetical by the second author's name. In three-or-more-author papers with the same first author, the order is chronological. The name of the author(s) should be followed by the date in parentheses, the full title of the paper together with the source of the reference, the volume number, and the first and last pages. The following illustrate the form to be used:

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Hodgkin AL, Huxley AF (1952b) The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. J Physiol (Lond) 116:497–506.

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Abbreviations

Nonstandard abbreviations should be introduced by placing the abbreviation in parentheses after the first occurrence of the term being abbreviated. The metric system should be used for all volumes, lengths, weights, etc. Temperatures should be expressed in degrees celsius (centigrade). Units should conform to the International System of Units (SI) (see *Handbook of Chemistry and Physics*).

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Authors are encouraged to submit a disk containing the final version of their manuscript once it has been provisionally accepted. Since nearly all manuscripts require some revision, disks should not be sent with the initial submission. The disk should be labeled with the date, the manuscript number, the first author's name, the file name, and the software and hardware used. The cover letter should include a statement, signed by any one of the authors, that the electronic file matches the hard copy of the manuscript. Any discrepancy between the disk and the hard copy will result in a delay of publication.

The 3½-inch disk size is preferred, although 5¼-inch disks can be accommodated. All sections of the paper except tables should be in a single file, in the order specified elsewhere in these Instructions. (Table files need not be included since tables will be typeset from the hard copy.) The following word processing software may be used: WordPerfect (versions 4.0 through 5.1), Microsoft Word (versions 4 and 5), Multimate (Advantage 2, 3.6, 4.0), and WordStar (3.31, 3.4, 4.0, 5, 6). The disk format must be IBM-compatible; Macintosh users should save the manuscript file as MS-DOS on an IBM DOS disk, and authors who use word processing software not listed here may save the manuscript file in ASCII form on an IBM-compatible disk.

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It is understood that by publishing a paper in *The Journal of Neuroscience* the authors agree, whenever possible, to make freely available to colleagues in academic research any clones of cells, nucleic acids, antibodies, etc. that were used in the research reported and that are not available from commercial suppliers.

Policy on DNA Sequences

In general, only complete genomic and cDNA sequences will be accepted for publication. The accuracy of the sequence must be confirmed by analyses of both strands. Sequences must be deposited in a data base generally accessible to the neuroscience community; the sequence accession number should be provided. Exceptions to this policy may be considered on an individual basis.

Policy on Animal Treatment

The Society for Neuroscience endorses the principles embodied in the Declaration of Helsinki concerning studies involving human subjects and the appropriate care and use of experimental animals. Therefore, all animal experimentation reported in the *Journal* must have been conducted in accordance with the following statement regarding the care and use of animals, approved by the Society for Neuroscience in April 1984:

Continued progress in many areas of biomedical research requires the use of living animals in order to investigate complex systems and functions because, in such cases, no adequate alternatives exist. Progress in both basic and clinical research in such areas cannot continue without the use of living animals as experimental subjects. The use of living animals in properly designed scientific research is therefore both ethical and appropriate. Nevertheless, our concern for the humane treatment of animals dictates that we weigh carefully the benefits to human knowledge and welfare whenever animal research is undertaken. The investigator using research animals assumes responsibility for proper experimental design, including ethical as well as scientific aspects.

The scientific community shares the concern of society at large that the use of animals in research should conform to standards that are consonant with those applied to other uses of animals by humans. Most of the more specific sections of this document were formulated with respect to research using warm-blooded vertebrates. As a general principle, however, ethical issues involved in the use of any species, whether vertebrate or invertebrate, are best considered in relation to the complexity of that species, its nervous system and its apparent awareness of the environment, rather than physical appearance or evolutionary proximity to humans.

Factors that relate to the design of experiments. The primary factor used to evaluate humane treatment in animal research is degree of distress or discomfort, assessed by anthropomorphic judgments made by reasonable and prudent human observers. *The fundamental principle of ethical animal research is that experimental animals must not be subjected to avoidable distress or discomfort.* This principle must be observed when designing any experiment that uses living animals.

Although most animal research involves minimal distress or discomfort, certain valid scientific questions may require experimental designs that inevitably produce these effects. Such situations, while uncommon, are extremely diverse and must be evaluated individually. It is critical that distress and discomfort be minimized by careful experimental design. It is also important to recognize that there is no difference between distress and discomfort that may be inherent in a valid experimental design and that which may occur as an unintended side effect. It is therefore incumbent on the investigator to recognize and to eliminate all *avoidable* sources of distress and discomfort in animal subjects. This goal often requires attention to specifics of animal husbandry as well as to experimental design.

Invasive procedures and paralytic drugs should never be employed without benefit of anesthetic agents unless there is a very strong scientific justification and careful consideration is given to possible alternatives. Advances in experimental techniques, such as the use of devices chronically implanted under anesthesia, can offer alternative approaches. If these are not feasible, it is essential to monitor nocioceptive responses (for example, recordings of EEG, blood pressure, and pupillary responses) that may indicate distress in the animal subject, and to use these as signals of the need to alleviate pain, to modify the experimental design, or to terminate the experiment.

When designing research projects, investigators should carefully consider the species and numbers of animals necessary to provide valid information, as well as the question whether living subjects are required to answer the scientific question. As a general rule, experiments should be designed so as to minimize the number of animals used and to avoid the depletion of endangered species. Advances in experimental methods, more efficient use of animals, within-subject designs, and modern statistical techniques all provide possible ways to minimize the numbers of animals used in research. This goal is completely consistent with the critical importance of replication and validation of results to true progress in science.

Factors that relate to the conduct of experiments. Research animals must be acquired and cared for in accordance with the guidelines published in the *NIH Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publications No. 80-23, Revised 1978). Investigators must also be aware of the relevant local, state, and federal laws. The quality of research data depends in no small measure on the health and general condition of the animals used, as well as on specifics of experimental design. Thus, proper animal husbandry is integral to the success of any research effort using living animal subjects. General standards for animal husbandry (housing, food quality, ventilation, etc.) are detailed in the *NIH Guide*. The experienced investigator can contribute additional specifics for optimum care for particular experimental situations, or for species not commonly encountered in laboratory settings.

Surgery performed with the intent that the animal will survive (for example, on animals intended for chronic study) should be carried out, or directly supervised, by persons with appropriate levels of experience and training, and with attention to asepsis and prevention of infection. Major surgical procedures should be done using an appropriate method of anesthesia to render the animal insensitive to pain. Muscle relaxants and paralytics have no anesthetic action and should not be used alone for surgical restraint. Postoperative care must include attention to minimize discomfort and the risk of infection.

Many experimental designs call for surgical preparation under anesthetic agents with no intent that the animal should survive. In such cases, the animals ordinarily should be maintained unconscious for the duration of the experiment. At the conclusion of the experiment, the animal should be killed without regaining consciousness and death ensured before final disposition.

Certain experiments may require physical restraint, and/or withholding of food or water, as methodological procedures rather than experimental paradigms. In such cases, careful attention must be paid to minimize discomfort or distress and to ensure that general health is maintained. Immobilization or restraint to which the animals cannot

be readily adapted should not be imposed when alternative procedures are practical. Reasonable periods of rest and readjustment should be included in the experimental schedule unless these would be absolutely inconsistent with valid scientific objectives.

When distress and discomfort are unavoidable attributes of a valid experimental design, it is mandatory to conduct such experiments so as to minimize these effects, to minimize the duration of the procedure, and to minimize the numbers of animals used, consistent with the scientific objectives of the study.

In submitting a paper to the *Journal*, each author will be assumed to have conscientiously abided by these principles. Animal welfare is a paramount concern in reviewing the methods section of submitted manuscripts.

Policy on Ethics

Finally, it is expected that authors submitting papers to *The Journal of Neuroscience* will have conducted their work in strict accordance with the following statement on ethics approved by the Society for Neuroscience in November 1989:

The Society for Neuroscience believes that progress in understanding the nervous system materially benefits human welfare. It recognizes that such progress depends on the honest pursuit of scientific research and the truthful representation of findings. While recognizing that both scientific error and differences of interpretation are a natural part of the creative process, the Society affirms that misconduct, in the form of fabrication, falsification, or plagiarism, jeopardizes the success of the entire scientific endeavor. Members of the Society assume an obligation to maintain the highest level of integrity in all scientific activities.

The primary responsibility for considering and resolving allegations of scientific misconduct lies within the individual academic communities and institutions where scientific work is carried out. The Society for Neuroscience therefore supports the principle that academic institutions should develop and have in place procedures to deal with allegations of scientific misconduct. However, the Society has a special responsibility and interest surrounding those scientific activities for which it is directly responsible, publication of the *Journal of Neuroscience* and the presentations at the annual meeting.

Every author of articles or abstracts submitted for publication in the *Journal of Neuroscience* or the *Neuroscience Abstracts* agrees to assume full responsibility, within the limits of his or her professional competence, for the accuracy of the report. In the case of multiple-authored papers, each author should have made a significant intellectual or practical contribution to the scientific work; "honorary authorship," i.e., the granting of authorship to persons who have made no substantive contribution to a scientific report, is not appropriate.

Scientists must have access to their original research results. The retention of accurately recorded and retrievable results is essential for the progress of scientific inquiry. Moreover, errors may be mistaken for misconduct when primary results are unavailable. Primary data should remain in the laboratory and should be preserved as long as there may be a reasonable need to refer to them.

Authors submitting articles or abstracts do so with the understanding that reports have not been submitted elsewhere. An abstract is a proper forum for rapid communication of work that will subsequently appear as a full-length article. However, submission of abstracts reporting already published work or publication of multiple similar manuscripts or abstracts, i.e., duplicate publication, is improper. When previously published data are presented as part of a new manuscript or abstract, as in a gradually developing longitudinal data set, or if a subject group or condition is included again for comparison purposes, citations to the previously published work should appear explicitly in the new report.

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Questions raised about the conduct of experiments or their presentation will be evaluated preliminarily by the Editor of the *Journal of Neuroscience* (in the case of an article in the *Journal*) or by the Chair of the Program Committee (in the case of an Abstract), in consultation with the Chair of the Publications Committee and the Secretary of the Society. If possible, the matter may be resolved informally at this level. However, if deemed appropriate, the matter will be referred to the institution where the scientific work in question was done. There, it would be expected that the matter would be reviewed in accordance with institutional procedures for handling allegations of misconduct. At all stages, every effort should be made to insure that the process is fair and just, both for those who are accused of misconduct and for those who have raised the issue of scientific misconduct.

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In the event that a published article or abstract is to be retracted, a statement of retraction will be published in the *Journal of Neuroscience* or in the *Abstracts* for the next annual meeting.

Rundown of NMDA channels during prolonged whole-cell recording in cultured 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 $[Ca^{2+}]o$ and with 2.4 mM BAPTA and 0.4 mM Ca^{2+} in the whole-cell pipette, the response evoked by regular applications of NMDA (10 μM) gradually decreased during prolonged whole-cell recording. After 25 minutes the peak current was reduced to 56 ± 1.6 % 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 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 minute 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 NMDA (300 μM) or when EGTA (10 mM) 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 42.8 ± 2.9 nS before, and 43.7 ± 2.8 nS after, rundown. The mean open times were 17.3 and 4.0 ms before rundown, 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 allow 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 an 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 et al., 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-methyl-4-

isoxazole proprionate) receptors is dependent on cAMP dependent protein kinase (Greengard, Jen & 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 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 phosphorylation. 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 anesthetized with halothane and killed by decapitation. Glass coverslips were coated with collagen (Vitrogen, Collagen Corp., USA) and poly-L-lysine (mol wt. 30-70 kDa, 10^{-5} M in 0.15 M borate, pH 8.4, Sigma, USA). Confluent astrocyte feeder layers were prepared by adding hippocampal cells (2.5×10^4 cells per $cm^{2)}$ 10 days before plating of neurons. For preparation of dissociated neurons, hippocampi were dissected free of meninges, cut in small pieces, and slowly agitated for one hour 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 $5\text{-}20 \times 10^3$ 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 Instr., USA). Patch pipettes were fabricated from borosilicate glass (TWF 150, WPI, USA) pulled with a conventional two-step puller (Sutter Instr.). Pipettes had 'bubble numbers' ranging from 7.2 to

8.0. After firepolishing, the inner pipette tip diameter was 1.5 to 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 mOsm. The ATP regenerating solution (Forscher & Oxford, 1985, MacDonald et al., 1989) including 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, Cs-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 insure stable cell access, the capacitance was monitored with a 7.5 ms hyperpolarizing voltage pulse (-10 mV) preceding each agonist application. Data was acquired using a split time clock with a 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 high MW reagents were used, and the time between formation of a GΩ seal and membrane rupture was delayed for 3 minutes 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 mm Hg) through the suction outlet controlled by a pressure transducer (Biotek, USA). Substitution of K^+ with the the impermeant cation N-methylglucamine (MW 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 ≈ proportional to $(MW)^{1/3}$, this suggests that the 2-3 minutes of whole-cell recording before the start of agonist application was sufficient to allow near complete exchange of reagents with MW < 1000, assuming no binding within the cell (Pusch & Neher, 1988), whereas a reagent with a MW = 100 kD would have an approximate exchange time constant of 5 minutes. Most intracellular reagents used were in this range except for several enzymes such as alkaline phosphatase (MW 80 kD).

Drug delivery

The extracellular medium contained (mM): NaCl, 167; KCl, 2.4; HEPES, 10; glucose, 10 and $CaCl_2$, 1 and glycine .01. The agonist solution contained 10 μM NMDA and either 2 mM or no added Ca^{2+} . The osmolarity was 325 mOsm and the pH 7.2. TTX (500 nM), picrotoxin (100 μM) and strychnine (2 μM) 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 ± 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 nonspecific 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 NMDA (10 μM) was applied every 30 s for 3 s. Membrane currents were recorded using PCLAMP (V.5.5, Axon Instr, USA) on an IBM-compatible computer and analyzed on a MacIntosh using AXOGRAPH software (Axon Instr. 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 minutes to the current evoked 2 minutes after the beginning of whole-cell recording. Data is expressed as percent of control ± SE. Significance was tested using one-way analysis of variance with the Bonferroni-Dunn procedure for multiple comparisons. P levels are given for significance levels were ≤ 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 NMDA (10 μM) every 15 s in 0.2 mM 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

Reagent grade chemicals were obtained from the following sources (square brackets indicate solvent used for stock solution, if other than H_20): Calbiochem, USA - K_2-ATP, K_2-creatine phosphate, okadaic acid [dimethylformamide], LR-microcystin; Sigma (USA) -phosphocreatine kinase/rabbit muscle, calmidazolium [dimethyl sulfoxide, DMSO], staurosporine [DMSO], Na-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. Cs_4-BAPTA, was obtained from Molecular Probes (USA). MK-801 [ethanol] was a gift of 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); 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 I, 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 NMDA (10 μM) in the absence (left panel) and presence (right panel) of ATP regenerating solution are shown in Figure 1A. Responses were evoked by 3 second applications of NMDA applied at 30 second 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 minutes of recording (n=9). The rundown of the peak current was accompanied by a loss of the slow relaxation ('inactivation') during the 3 second NMDA application (Fig 1A, left panel). 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 minutes (103.2 ± 1.6 %, t=25 minutes; n=10, $p<.0001$). However, Ca-dependent inactivation was not prevented by the ATP regenerating solution (Fig 1A, right panel).

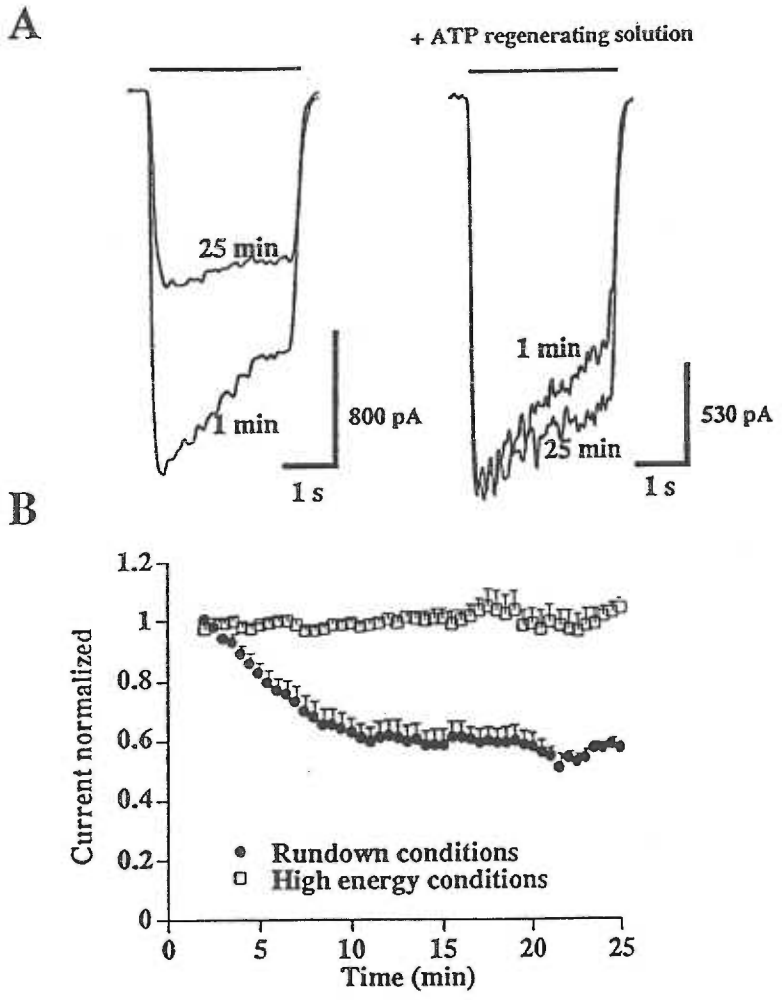

Figure 1. *Rundown of NMDA currents during prolonged whole-cell recording was prevented by ATP regenerating solution.*
Inward currents were evoked by 3 s applications of 10 μM NMDA at 30 s intervals. A. Currents evoked after 1 and 25 minutes of whole-cell recording are superimposed in the absence (left) and presence (right) 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.

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 ~6 minutes (Fig. 1B). 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. 1A, right panel). 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 two minutes 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 (107.6 ± 5.6 %, 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. 2A). In addition, no significant current rundown occurred in Ca^{2+}-free solutions even in the absence ATP regenerating solution (99.4 ± 3.1 %; n=9, $p<.0001$; Fig. 2A). 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

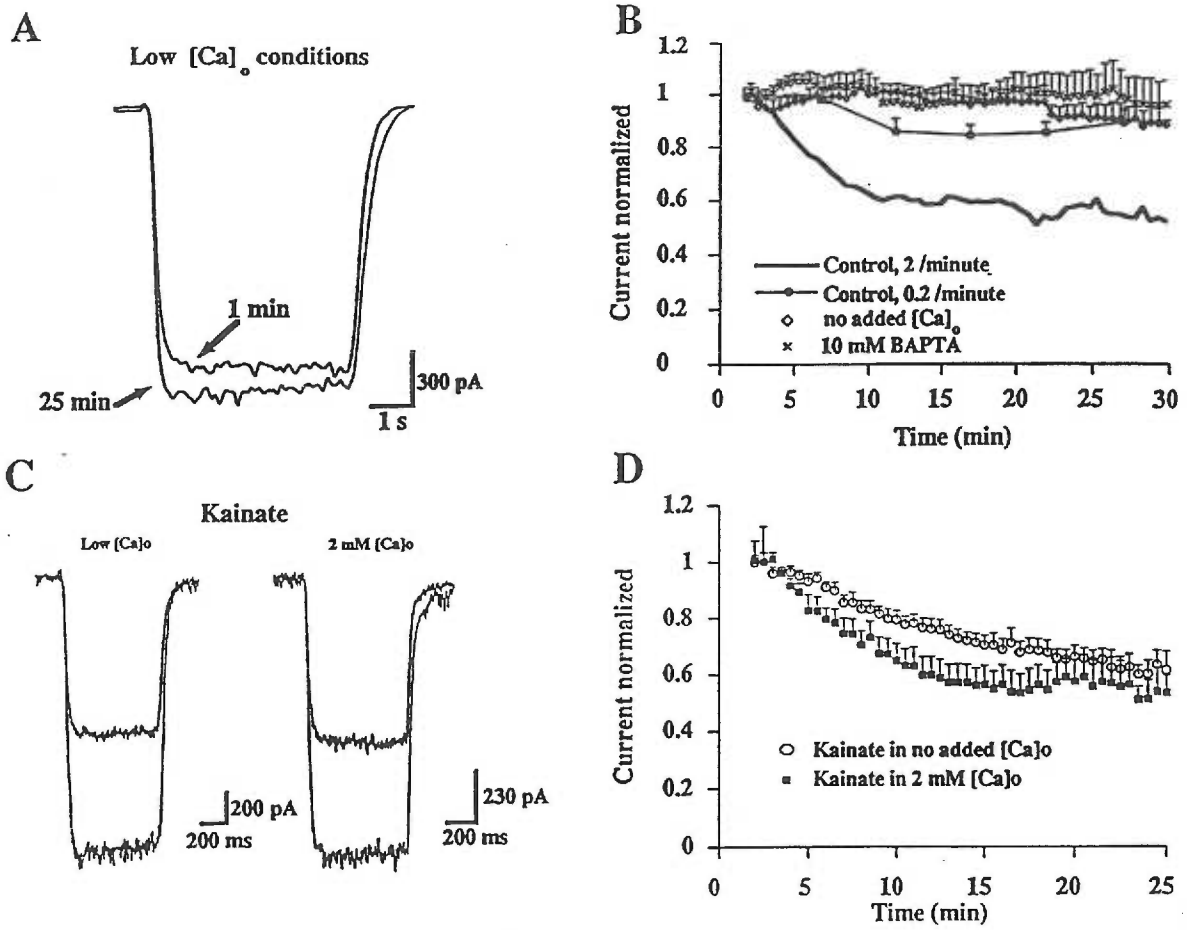

Figure 2. *Rundown of NMDA channels was Ca^{2+}-dependent*
A. Inward currents evoked by NMDA (10 μM) in $[Ca^{2+}]o$-free solution are superimposed for one neuron at 1 and 25 min of whole-cell recording in the absence of added ATP regenerating solution ("low [Ca]o conditions"). B. NMDA currents, evoked 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 (black line) are reproduced from Figure 1 for comparison. C,D. Rundown of kainate currents was not dependent on $[Ca^{2+}]o$. Currents evoked by kainic acid (20 μM) at 1 minute and 25 minutes in either low $[Ca^{2+}]o$ or 2 mM $[Ca^{2+}]o$ are shown. Normalized kainate responses (n=7) are shown in D.

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 or more minutes, 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 minutes in Ca^{2+}-containing solutions, the rundown was nearly abolished (90.1 ± 5.8 %, n=5, $p<.0001$). Rundown was also reduced when BAPTA was increased to 10 mM in the patch pipette (95.8 ± 3.8 %, n=6, $p<.0001$) . These results are summarized in Fig. 2B. The Ca^{2+}-dependence of NMDA channel rundown was receptor-specific as rundown of kainate responses was similar in the absence (61.7 ± 4.8%) and presence (52.6 ± 8.4%) of extracellular Ca^{2+} (n=7, Fig. 2C, 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 of transmembrane calcium flux through NMDA channels. As shown in Fig. 3A, responses evoked by NMDA (10 μM) in Ca^{2+}-free solution were stable during the first 10 minutes of recording with 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 seven minutes to 67.4 ± 4.9 % (n=4). The inhibition was reversible on reperfusion with control buffer (92.5 ± 5.2 %, 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 3 neurons, 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. 3B).

ATP might prevent rundown by either facilitating the clearance of calcium (Byerly & Moody, 1984; Miller, 1991) or neutralizing its effect. To examine the relationship between Ca^{2+} and ATP, we tested whether intracellular perfusion with ATP regenerating solution was sufficient to reverse rundown (Fig. 3B). Rundown, induced by switching from Ca^{2+}-free to a Ca^{2+}-containing agonist solution, reached 64.5 ± 3.6 % (n=4) after 5 minutes. Subsequently, intracellular perfusion with ATP regenerating solution resulted in full recovery (94.6 ± 4.7 %, Fig. 3B) despite the continued agonist applications. Thus ATP is sufficient to overcome Ca^{2+}-dependent rundown.

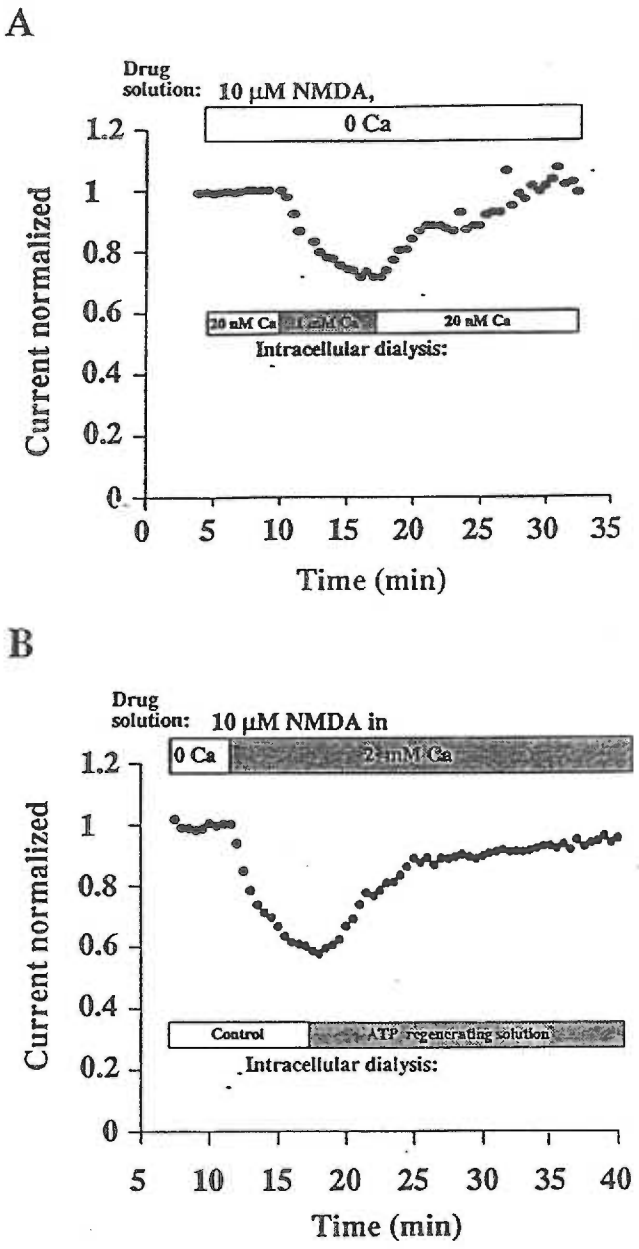

Figure 3. ***Intracellular perfusion with high Ca^{2+} produced reversible inhibition of the NMDA current and ATP counteracted Ca^{2+}-dependent rundown .***
A. Currents were evoked under low external calcium conditions. The cell interior was then perfused for 7 minutes with a solution containing 1 mM 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.

The Na-Ca 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 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 $[Na^+]o$ and 2 mM $[Ca^{2+}]o$, and ATP regenerating solution was included in the pipette (Fig. 4A). After a 2 minute baseline period, the neurons was 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 two minutes in low $[Na^+]o$, and completely recovered on return to 167 mM sodium (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 second interpulse interval, as is the case for the cell shown in Figure 4A. 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 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 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 exchanger with 100 μM bepridil also produced a reversible decrease in the current (67.7 ± 5.1 %, n=5, Fig. 4B). 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 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.

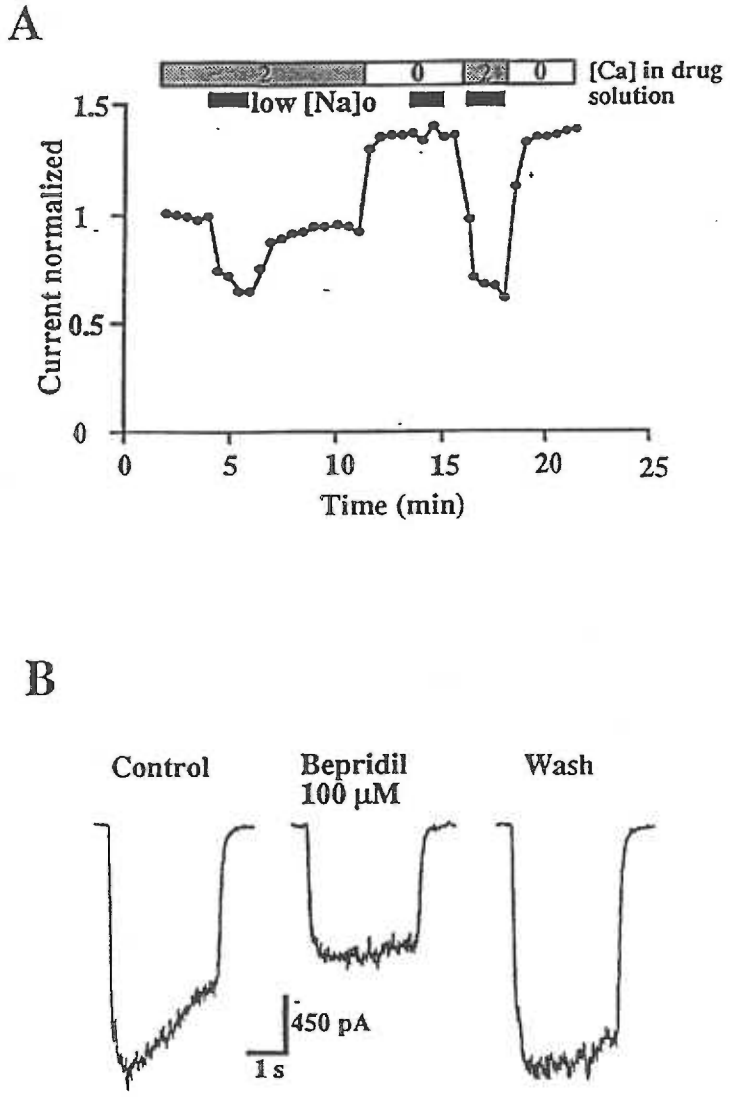

Figure 4. *The Na-Ca exchanger is required for maintenance of NMDA currents .*

A. NMDA currents were evoked in a neuron under high energy conditions as outlined in Figure 1. Reduction in $[Na^+]o$ to 42 mM during the 27 s intervals between NMDA applications (black 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 exchanger with bepridil (100 µM) also produced a 50 % inhibition after five NMDA applications that was fully reversible 8 minutes after washout of bepridil.

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. 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 Figure 5A, 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 minutes. Figure 5B shows superimposed responses of two cells that ran down to 60 % of control after 30 minutes 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 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 favored 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/minute, 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 minutes of intense stimulation (5-10 applications of NMDA (1 mM), duration 5 s each). The protease inhibitors leupeptin (100 μM) and calpain inhibitor 1 (50 μM) were added to the patch solution. We compared three buffer conditions: EGTA (10 mM with no added Ca^{2+}) in the presence or absence of ATP regenerating solution, and BAPTA (2.4 mM/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 ≈50 % of control (Fig. 5C). 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 5C). 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.5C,D). This suggests that rundown becomes irreversible when the Ca^{2+} transient exceeds a certain threshold.

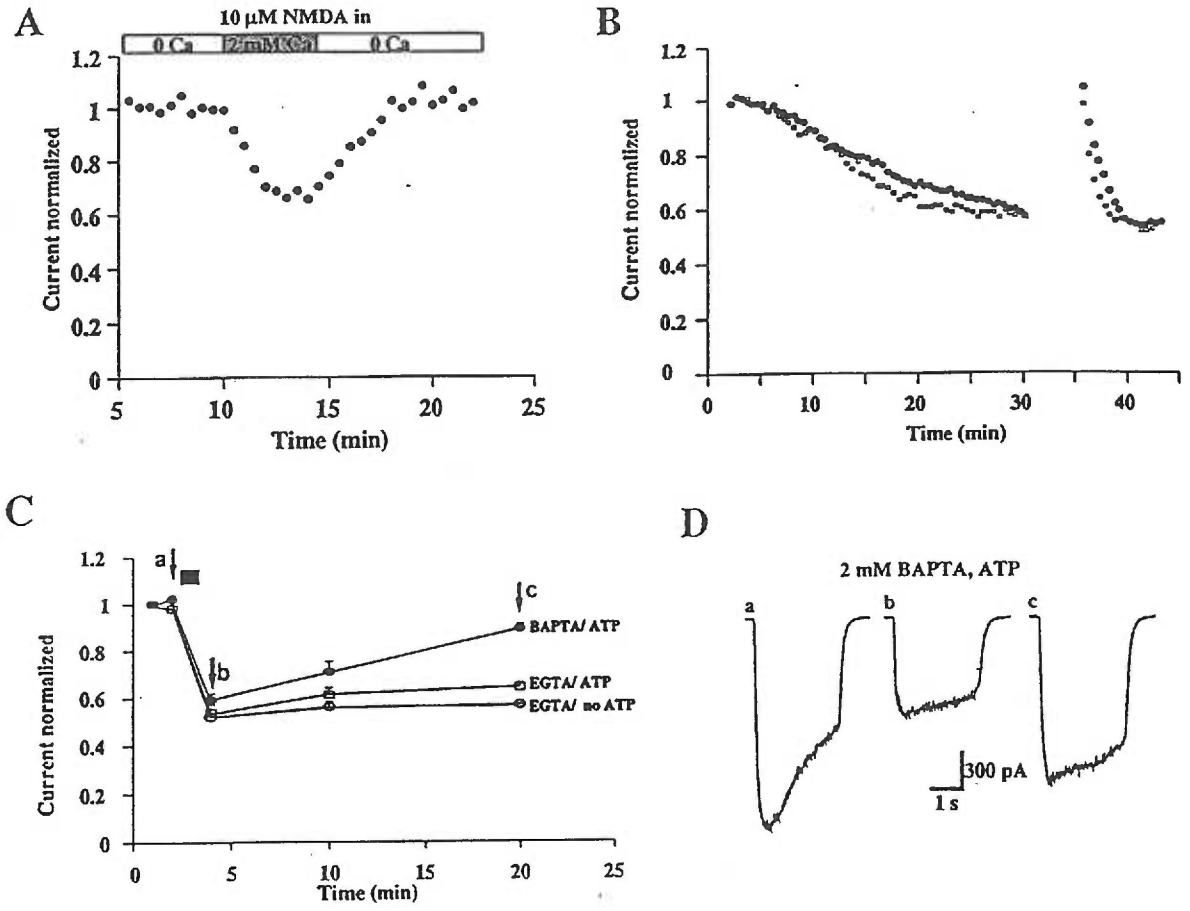

Figure 5. *The reversibility of channel rundown was condition dependent.* A. In the absence of ATP regenerating solution, currents evoked by NMDA were reversibly inhibited when extracellular [Ca^{2+}] was increased to 2 mM for 5 minutes. The currents in 2 mM [Ca^{2+}]o were normalized. B. Rundown of two neurons dialyzed with control buffer and 2 mM Ca^{2+} in the agonist solution are superimposed. After agonist applications were suspended for 5 minutes, 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 5-10 five second pulses of 1 mM NMDA in 2 mM [Ca^{2+}] for 1-2 minutes (black bar). Subsequent NMDA responses after 1, 11 and 21 minutes recovered for cells dialyzed with BAPTA and ATP regenerating solution, but not for cells dialyzed with EGTA in the presence or absence of ATP regenerating solution. Leupeptin (100μM) and calpain inhibitor 1 (50 μM) were included in the patch solution. D. Responses for one cell dialyzed with BAPTA are shown at times indicated by a, b and c in C.

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 paradigms 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 minutes 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 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).

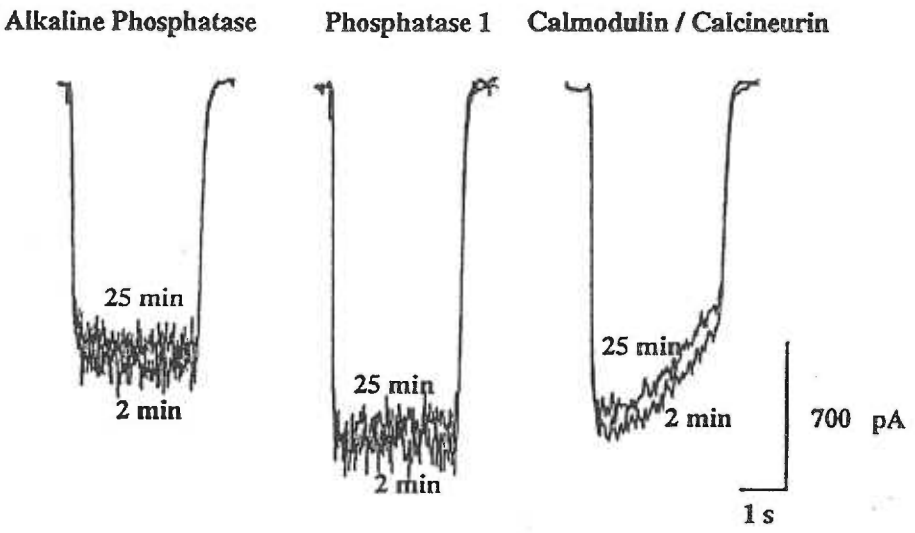

Figure 6. ***Intracellular dialysis with phosphatases did not inhibit NMDA currents.***
Currents evoked by NMDA at 2 and 25 minutes of whole-cell recording are superimposed for 3 neurons. Alkaline phosphatase (100 µg/ml), phosphatase 1 (250 ng/ml) or or calcineurin (120 U/ml) in combination with calmodulin (600 U/ml) had no effect on the peak amplitude of the current. For alkaline phosphatase and phosphatase I, 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.

ATP could prevent rundown by mechanisms other than phosphorylation. For example, the nonspecific ATPase inhibitor vanadate (10 μM) rapidly inhibited NMDA currents under high energy conditions (77.0 ± 7.5 %, n=7, p<.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. 7A). However, rundown was significantly delayed by phosphate. After 8 minutes of whole-cell recording, the response was 89.6 ± 3.1 % (p<.001) for 25 mM phosphate and 88.5 ± 6.3 % (p<.001) for 125 mM phosphate compared to 68.2 ± 5.1 % in the absence of phosphate or ATP regenerating solution (Fig 7B). 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<.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 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<.0001) than ATP regenerating solution. Addition of 4 mM ATP-γ-S to the ATP (4 mM) 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.

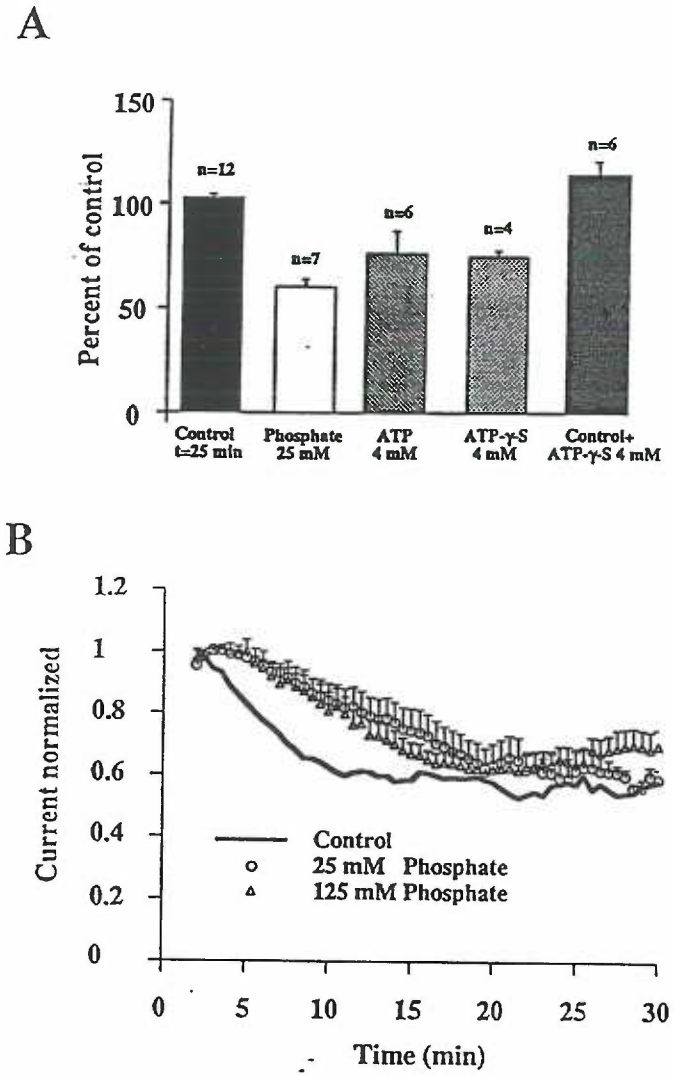

Figure 7. *The efficacy of different phosphates in preventing rundown*
A. Cells were dialyzed for 25 minutes with different phosphates. NMDA currents (mean ± SE) at 25 minutes were normalized to the peak current after 2 minutes of recording. Phosphate (25 mM) did not prevent rundown. ATP (4 mM) as well as ATP-γ-S (4 mM) 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 dialyzed with 25 or 125 mM phosphate. Rundown in the absence of phosphates (control) is reproduced from Figure 1 for comparison (black line).

To further evaluate 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. The activity of the reagents was tested in biochemical assays, concentrations used in the pipette were supramaximal, and 2-3 minutes 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, $p<.0001$) after 25 minutes 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-K II inhibitory peptide 281-302/A286 (50 μM, Soderling, 1990) in a concentration that fully blocks CaM-K II had no significant effect (93.8 ± 3.6 %, n=7). The catalytic subunit of protein kinase A (9 μg/ml, n=4) or phorbol-D-butyrate (100 nM) also did not increase the response above control levels. The current amplitude at 25 minutes 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 minutes was 65.6% ± 5.1, (n=5) in the presence of okadaic acid (5 μM), 60.5 ± 3.3% (n=5) in the presence of LR-microcystin (10 μM), and 61.1 ± 3.0 (n=5) in the presence of CaN inhibitory peptide A457-491 (100 μM, Hashimoto, Perrino & Soderling, 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 Ba^{2+} (2 mM) was substituted for Ca^{2+}(89.4 ± 2.1 %, n=12, p <.0001), and the calmodulin inhibitor calmidazolium (CMZ, 10 μM) significantly retarded rundown (83.6 ± 4.5 %, n=11, p <.0001). CMZ also appeared to slightly increase 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 Po underlies rundown

Ca^{2+}-dependent rundown is not apparent in outside-out membrane patches (Sather et al., 1990; Rosenmund and 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,

$$I_{wc} = n*i*MOT*P_O$$

where n is the number of available channels, i is the single channel current, MOT is the mean open time, and Po is the open probability. Po 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 8A).

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 NMDA (10 μM) 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 current (Fig 8B). 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 5 neurons the conductance before rundown was 42.8 ± 2.9 pS, consistent with prior estimates of γ by fluctuation analysis (Mayer et al, 1988). Following rundown there was no significant change in the conductance (43.7 ± 2.8 pS). The MOT was estimated by fitting the power spectra with the sum of two Lorentzians (Fig 8C). Before rundown the corner frequencies (f_{c1} and f_{c2}) were 40.5 ± 2.2 and 9.2 ± 0.9 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 40.1 ± 1.1 and 10.0 ± 0.7 Hz with time constants of 4.0 and 15.9 ms.

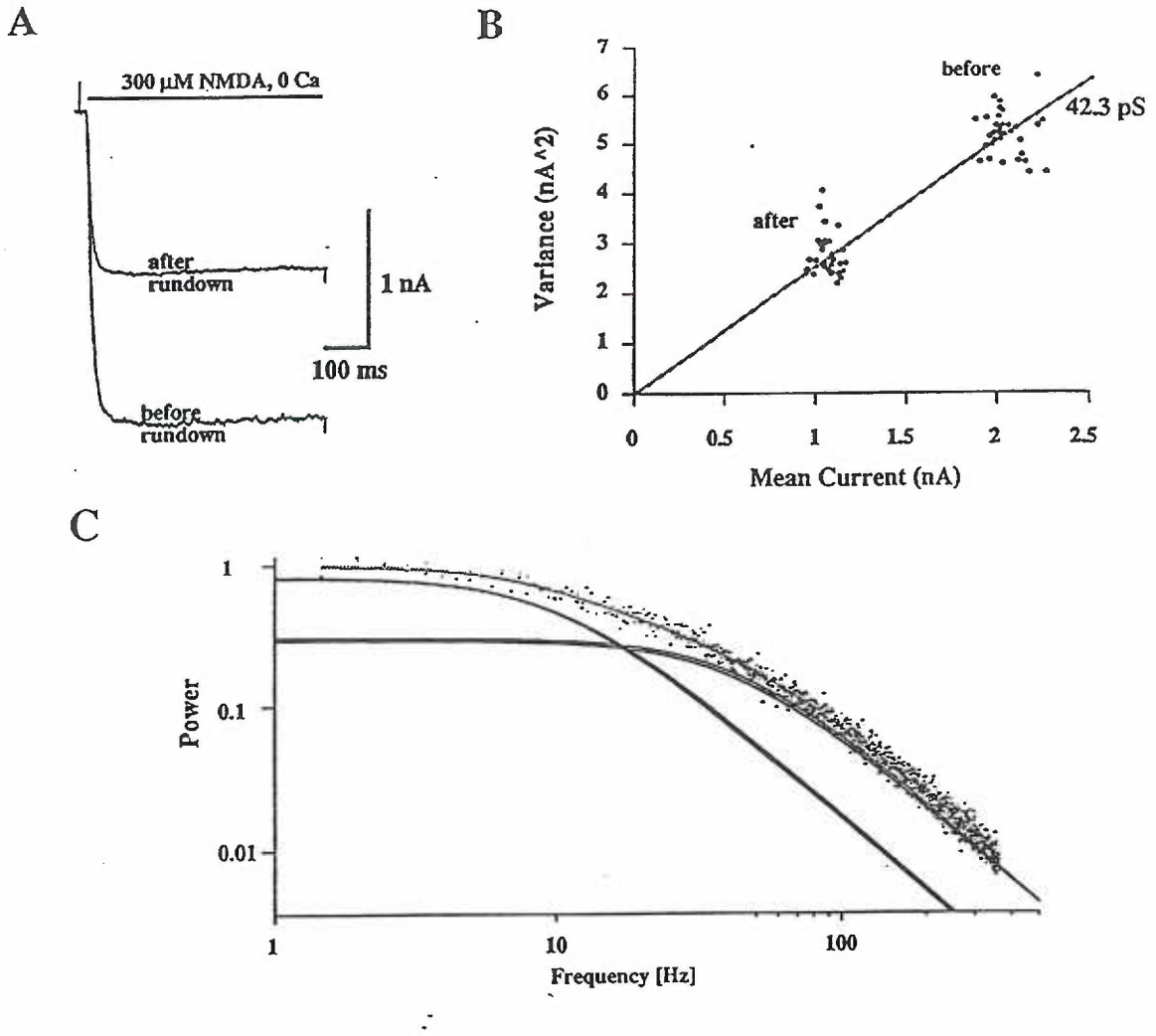

Figure 8. *Rundown did not result from a decrease in agonist affinity or channel lifetime.*

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 NMDA pulses (10 µM) in 0.2 mM [Ca^{2+}]o was used to calculate the mean-variance relationship before and after rundown. The dotted 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.

By exclusion, the results of fluctuation analysis suggest that rundown is due to either a change in the number of channels or in Po. To estimate these parameters, we analyzed the open channel block of whole-cell currents by MK-801. The irreversible block by MK-801 ($\tau_{recovery} \approx 90$ minutes, Huettner & Bean, 1988) has been used to estimate the Po 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 Po 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 paradigm is shown in Fig. 9A. After the current evoked by NMDA in a low extracellular calcium (0.2 mM) reached steady state, MK-801 (20 μM) 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 ≈ 50% of the response at 10 μM NMDA (Fig. 10C), and thus the estimated Po 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 minute later showed no recovery (data not shown). The decay could be fitted with two exponentials. The initial decay had a time constant of 114.1 ± 10.4 ms, and a coefficient of 68.56 ± 0.64 % (n= 17), presumably reflecting the equilibration of MK-801 at the cell. The % 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 and Westbrook, 1991). However the slow decay was prolonged as the agonist concentration was decreased, i.e. as Po decreased. The decay in 5 and 10 μM NMDA for two cells are shown in Figure 9B. As expected, the decay was slower in 5 μM NMDA, but the number of channels as measured by the charge transfer (i.e. the area under the curve during the decay) was unchanged (Fig 9B; 10C, middle panel).

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

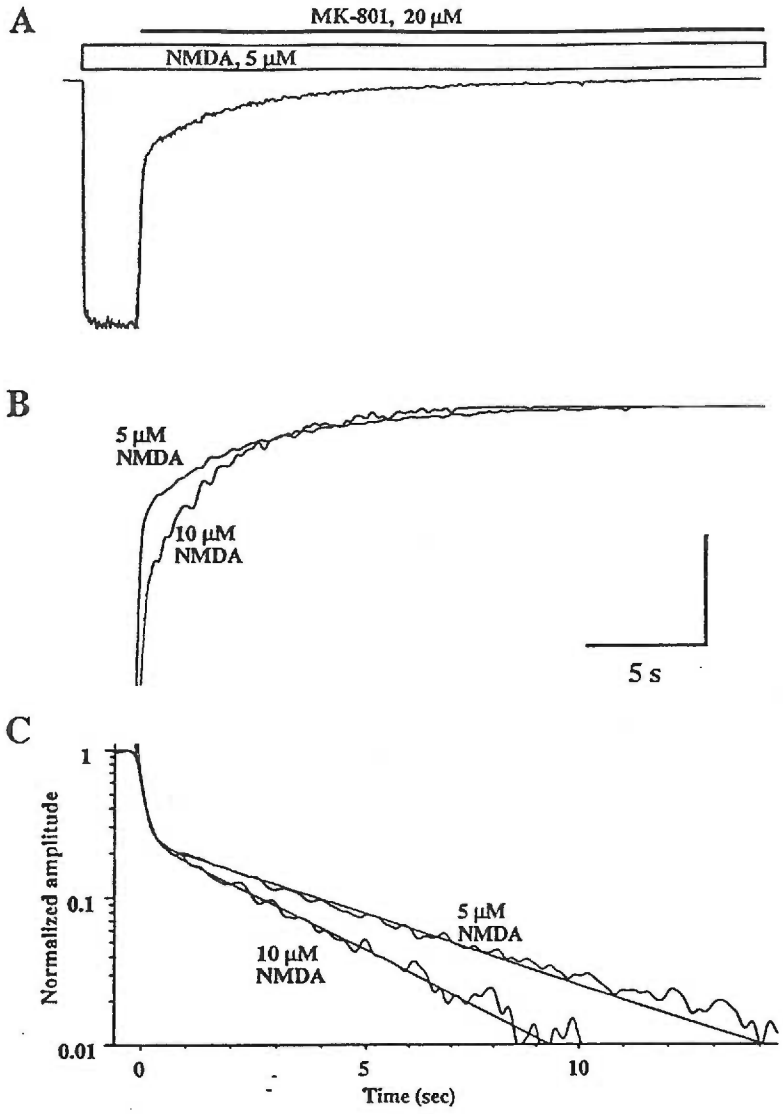

Figure 9. *Changes in open probability were measured by the time course of channel block by MK-801*

A. Current evoked by a two second application of NMDA (5 μM) decayed with fast and slow components when exposed to NMDA (5 μM) + MK-801 (20 μM). 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 NMDA (10 μM). The two neurons had comparable steady state currents evoked by NMDA (10 μM). 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%). C. Semilogarithmic plot of the current decays in MK-801 with superimposed exponential curve fits are shown for the neurons in A and B.

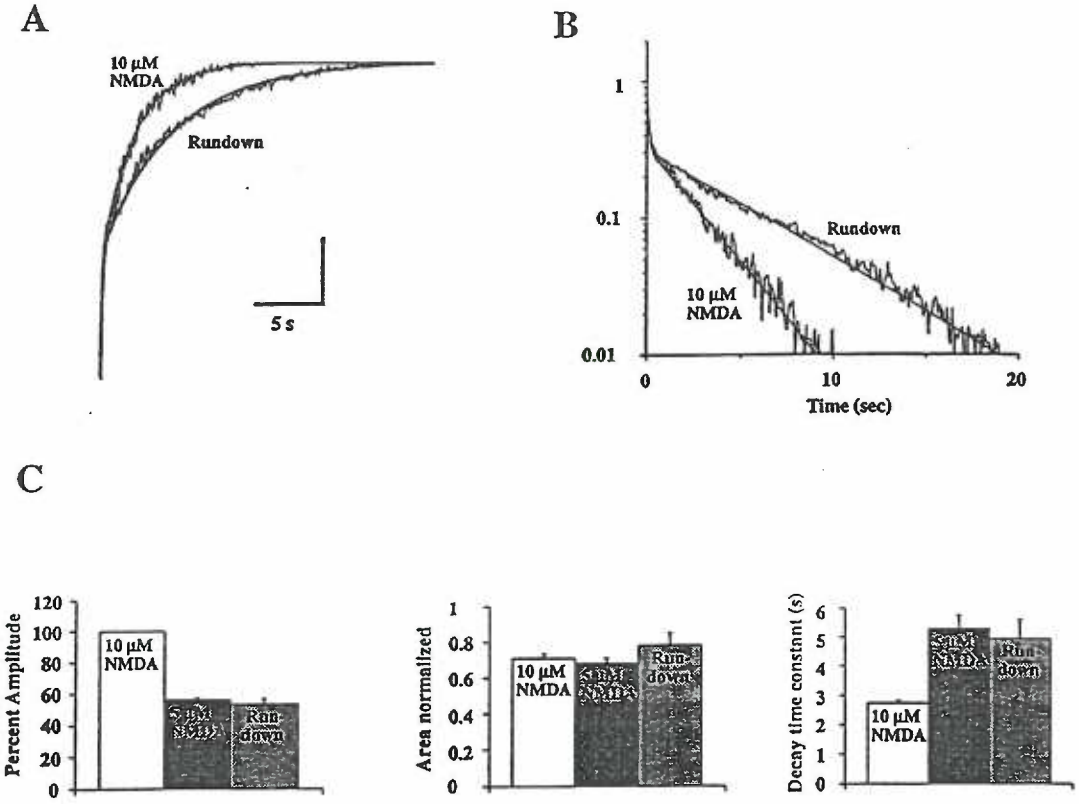

Figure 10. *Rundown resulted from a reduction of the open probability.* A. The decay of the current following solution exchange from NMDA (10 μM) to NMDA + MK-801 (20 μM) are superimposed for a neuron before rundown ("10 μM NMDA"), and for a second neuron after rundown ("Rundown"). The currents were normalized to the steady state current immediately preceding exposure to MK-801; calibration was 130 pA and 70 pA, respectively. The best fit to the sum of two exponentials are 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 (left panel), charge transfer during MK-801 block (expressed as nC per unit steady state current of 1 nA, middle panel), and the slow time constant (right panel) 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 constants under these conditions were 2.74 ± 0.08 s (coefficient 31.1 ± 2.3 %), 5.3 ± 0.5 s (coefficient 30.8 ± 1.9%) and 5.0 ± 0.7 s (coefficient 32.3 ± 1.2 %), respectively.

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 ≈50% during recording periods up to 45 minutes. 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 behavior. 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-containing solutions and NMDA in Ca-free solutions did not lead to rundown of the NMDA channel (not shown). If voltage escape had occurred, Ca loading during KA 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 MW test reagent (N-methylglucamine, MW 195, see Methods).

Based on this rate (e.g. 90 s complete exchange time for N-methylglucamine), we futher delayed the beginning of each experiment (2-3 minutes) to allow diffusion of the higher MW 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 ~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 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 minutes 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 & Fischbach, 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 seconds (Legendre et al., 1993) . The whole-cell current decreased by 50% with a time constant of ≈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 an important factor (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 (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 that phosphorylation of a regulatory protein contributes to maintenance of the current. However, this could also be nonspecific, 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 exchanger (Blaustein, 1988; Miller, 1991). ATP modulates the function of the Na-Ca exchanger, and functional Na-Ca exchange was required to prevent rundown in the presence of ATP regenerating solution. In squid axons, ATP ($K_d \approx 250$ μM) 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 and 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.

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CALCIUM-INDUCED ACTIN DEPOLYMERIZATION REDUCES NMDA CHANNEL ACTIVITY.

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Summary

Actin filaments are highly concentrated in postsynaptic densities at central excitatory synapses, but their influence on postsynaptic glutamate receptors is unknown. We tested whether actin depolymerization influences NMDA channel activity in whole-cell recording from cultured hippocampal neurons. The ATP- and calcium-dependent rundown of NMDA channels was prevented when actin depolymerization was blocked by phalloidin. Rundown of kainate responses was unaffected by phalloidin. Cytochalasins, which enhance actin-ATP hydrolysis induced NMDA channel induced rundown, whereas the addition of microtubule reagents taxol and colchicine, or protease inhibitors, had no effect. Our results suggest that calcium and ATP can influence NMDA channel activity by altering the state of actin polymerization, and are consistent with a model in which actin filaments compartmentalize an essential regulatory protein.

Introduction

Synaptic activation elevates calcium in dendritic spines (Müller and Connor, 1991). One result of postsynaptic calcium influx is induction of long-term potentiation (Collingridge and Bliss, 1987). Although the cellular targets for calcium are still poorly defined, the components of the postsynaptic density (PSD) provide a number of potential candidates. The PSD consists of a dense matrix of regulatory proteins that is essentially an extension of the neuronal cytoskeleton (Gulley and Reese, 1981). In addition to a well established role in the compartmentalization and anchoring of membrane proteins (Bamburg and Bernstein, 1991), the neuronal cytoskeleton has also been suggested to play a role in synaptic plasticity (Fifková, 1985; Lynch and Baudry, 1987). The major cytoskeletal element in PSDs and dendritic spines is actin (Matus et al., 1982). Filamentous actin is under dynamic regulation, being rapid depolymerized by calcium while repolymerization requires ATP and other factors (Pollard and Cooper, 1986; Bennett and Weeds, 1986). Thus actin dynamics could affect postsynaptic functions.

Cytoskeletal elements are known to affect the localization of ion channels. For example, cytoskeletal proteins are involved in the clustering of nicotinic acetylcholine receptors at neuromuscular junctions (Froehner, 1991), and voltage gated sodium channels in axons (Srinivasan et al., 1988). The gating of mechanosensitive channels and transduction channels in hair cells has been suggested to involve an interaction with the cytoskeleton (Guharay and Sachs, 1984; Hudspeth,1989). Although glutamate receptor activation and subsequent calcium entry has been reported to produce changes in fodrin (brain spectrin) and microtubule-associated proteins (Siman and Noszek, 1988; Bigot and Hunt,

1990), it is unknown whether such changes alter the function of postsynaptic receptors.

NMDA (N-methyl-D-aspartate) and AMPA(α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate)/kainate receptors are colocalized at central excitatory synapses (Bekkers and Stevens, 1989). The regulation of these receptors has recently been probed by studying the 'rundown' of channel activity during whole-cell recording (MacDonald et al., 1989; Wang et al., 1991). For both receptors, the addition of ATP to the whole-cell pipette prevents rundown. For AMPA/kainate channels this has been linked to phosphorylation by protein kinase A (Greengard et al., 1991; Wang et al., 1991), however rundown of NMDA channels appears to involve a mechanism other than receptor phosphorylation (Rosenmund et al., 1991, Soc.Neurosci.Abstr. 17, 957). In addition, intracellular calcium inactivates the NMDA channel, and transmembrane calcium entry is required for channel rundown (Rosenmund et al., 1991, Soc. Neurosci. Abstr. 17, 957; Legendre et al., 1993). Because ATP and calcium have opposing effects on actin dynamics, we tested whether NMDA channel rundown is a direct result of changes in the actin cytoskeleton.

Currents were evoked by NMDA in whole-cell and inside-out patches from rat hippocampal neurons in primary culture. As expected rundown was triggered by calcium influx during whole-cell recording, but NMDA channels in inside-out patches were calcium-insensitive, suggesting that a critical calcium-dependent constituent was absent in the excised patch. Whole-cell dialysis with phalloidin completely blocked rundown, while cytochalasins induced rundown, strongly suggesting that calcium-induced actin depolymerization underlies

rundown, and that the integrity of the cortical cytoskeleton plays an important role in regulating NMDA channel activity.

Results

Inhibition of NMDA channels by intracellular calcium is restricted to the intact cell.

Figure 1A shows the calcium-dependence of whole-cell currents evoked by NMDA (10 μM) in a cultured hippocampal neuron. During the first few minutes of whole-cell recording, the agonist-evoked current reached an initial peak and then decayed to a steady state level of approximately 50%. We have previously shown that inactivation is due to increases in intracellular calcium (Legendre et al., 1993). During prolonged whole- cell recording (e.g. 25 minutes), the peak current gradually ran down to approximately the same steady state level as occured with inactivation (58.8 ± 1.8 % of control, n=10, Figure 1A, left panel). Channel rundown as well as inactivation were eliminated if agonist was applied in calcium-free solutions (96.8 ± 3.5 %, n=11; Fig 1A right), thus intracellular calcium is also required for rundown (Rosenmund et al., 1991, Soc. Neurosci. Abstr. 17, 957).

We first tested the possibility that intracellular calcium modulates channel activity by directly binding to the channel by using excised inside-out patches from cultured hippocampal neurons. Agonist was added to the patch solution; EGTA and EDTA (both at 5 mM) were added to the pipette to prevent Ca^{2+} influx or Mg^{2+} block of channels in the patch. The patch was alternately exposed for 500 ms to solutions containing either calcium(1-1000 μM) or no calcium (0 Ca^{2+}, 10

mM EGTA). The channel activity for 6 trials is shown in Figure 1B for a patch exposed to 100 μM calcium. Channel activity, determined from amplitude histograms for 50-100 trials was unaffected by exposure to calcium. In the presence of 1000 μM calcium the open probability was 98.3 ± 2.1 % (n=5) of that in calcium-free solution. The arithmetic mean open time and channel conductance were also unaffected. The mean open time for large conductance openings (>34 pS) was 3.4 ± 0.2 ms in calcium-free and 3.5 ± 0.3 in 1000 μM calcium. The main large conductance level was 68.5 ± 2.1 pS and 69.6 ± 1.6 pS, respectively. Similar results were obtained in 5 patches exposed to 1 or 100 μM calcium. This suggests that the observed calcium-dependence of NMDA currents is not the result of direct binding to the channel.

Because intracellular calcium can modulate NMDA channels in cell-attached patches (Legendre et al., 1993), the action of calcium apparently requires the environment of the intact cell. One obvious possibility is that intracellular factors are washed out of the inside-out patch. However rundown did not occur even after 40 minutes of whole-cell dialysis if the agonist solution was calcium-free. Thus rundown appears to involve more than washout of soluble factors. In addition, stable channel activity in inside-out or cell-attached patches was dependent on using low negative pressures (1-5 mm Hg ,133-665 Pa) in seal formation. This suggested the possibility that membrane-cytoskeletal interaction might be involved in regulating channel activity, as occurs in mechanosensitive channels (Guharay and Sachs, 1984; Hamill and McBride, 1992).

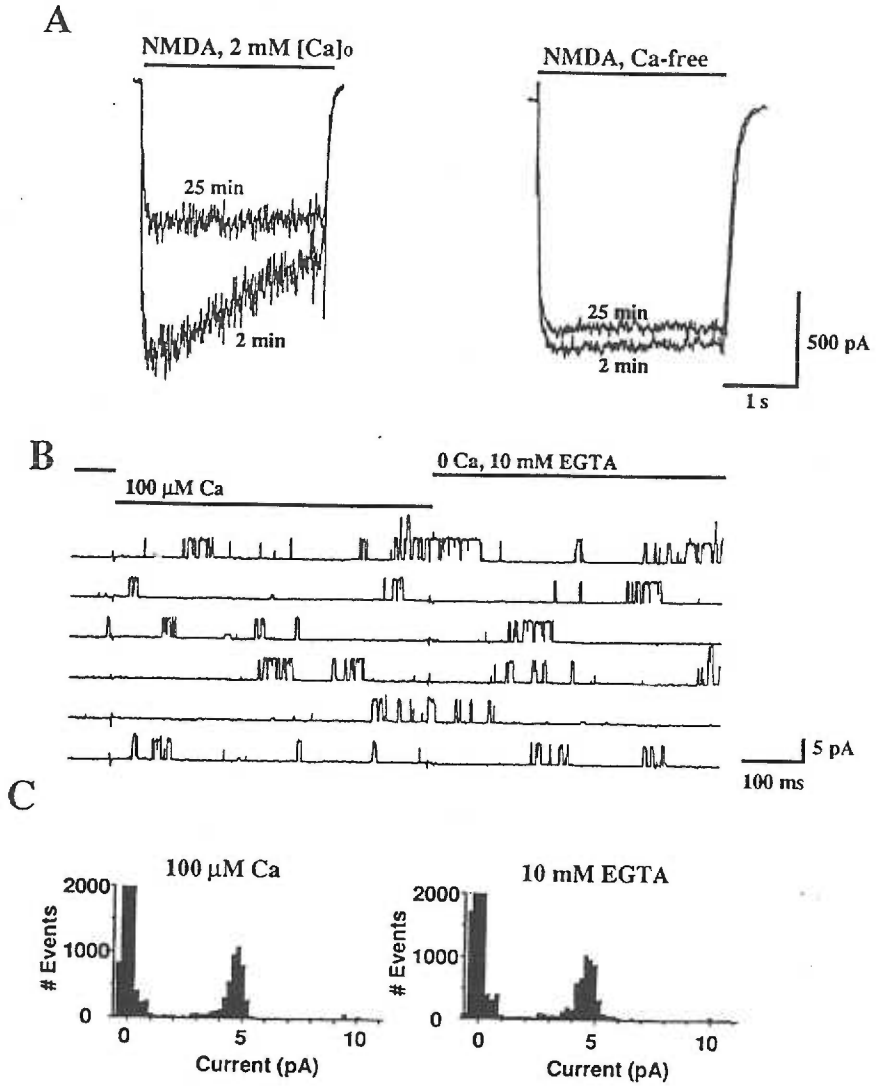


Figure 1. Inhibition of the NMDA channel by intracellular calcium is restricted to the intact cell.

A. Whole-cell currents (holding potential -60 mV) were evoked by timed application of NMDA (3 seconds; 2/minute). Low concentration of NMDA (10 μM) was used to prevent large, uncontrollable transmembrane Ca^{2+} influx. At 2 minutes the peak amplitude inactivated slowly, then ran down after 25 minutes of recording when the agonist solution contained 2 mM $[Ca^{2+}]o$ (left). In Ca^{2+}-free agonist solutions, no inactivation or rundown was apparent. The patch pipette contained 2.4 mM BAPTA, 0.4 mM Ca^{2+} and no ATP.

B. In inside-out recording, NMDA (100 μM) activated inward single channel currents of 4.6 pA at a holding potential of +65 mV. High (100 μM Ca^{2+}) and low (10 mM EGTA) calcium solutions were alternately applied to the inner side of the patch. Solution exchange is marked by the electrical artifact of the piezoelectric device.

C. Point by point amplitude histograms of the data ensembles for 50 trials in either high or Ca^{2+}-free conditions. The total open probability for this patch was 8.3% in EGTA and 7.9% in 100 μM Ca^{2+}.

Phalloidin blocks NMDA channel rundown

To test the hypothesis that calcium acts by altering the structural state of the actin cytoskeleton, we added the mushroom toxin phalloidin (1 μM) to the whole-cell pipette. Phalloidin binds with high affinity to filamentous actin and shifts the equilibrium between the actin polymer and monomers to the polymerized state (Cooper, 1987). Because phalloidin has a low diffusion coefficient (Pusch and Neher, 1988) due to a high capacity of binding sites, we dialyzed the cell for 3-5 minutes before applying NMDA in the presence of calcium. NMDA currents during the first minutes of recording, monitored using calcium-free agonist solutions, remained stable (not shown). When NMDA was applied in the presence of extracellular calcium, rundown was completely prevented by phalloidin (Fig. 2A). The current after 25 minutes of whole-cell recording was 101.5 ± 5.5 % (n=8, $p<.001$) of the initial amplitude (Fig. 2B) whereas in the absence of phalloidin the current ran down to 58.8 ± 1.8% (n=10). Phalloidin had no effect on the current when NMDA was applied in calcium-free solutions (97.4 ± 4.2 %, n=4). This suggests that actin depolymerization, triggered by calcium influx through the NMDA channel, leads to channel rundown. The effect was specific for NMDA channels as rundown of non-NMDA currents evoked by kainate (20 μM) was unaffected by intracellular dialysis with phalloidin. Kainate responses after 25 minutes ran down to 62.5 ± 4.7 % (n=6) in control and 68.4 ± 3.3 %(n=5) in the presence of phalloidin (Fig. 2B).

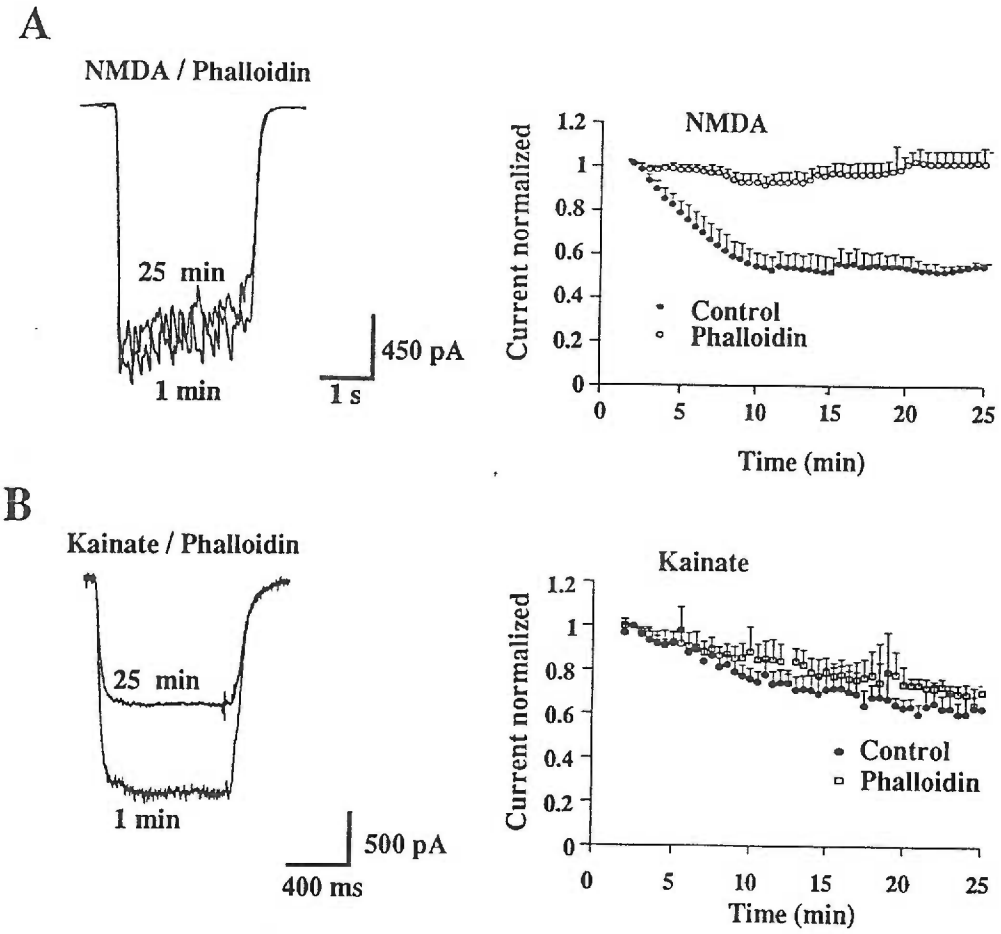


Figure 2. Calcium-dependent rundown of NMDA channels is blocked by inhibiting actin depolymerization.

A. Left panel shows responses evoked by NMDA (10 μM) for one neuron in presence of extracellular Ca^{2+} (2 mM) after 2 and 25 minutes of whole-cell recording . Phalloidin (1 μM), included in the whole-cell pipette, blocked channel rundown. Time course of NMDA currents evoked at 30 sec intervals in the presence and absence of phalloidin are shown in the right panel.

B. Phalloidin had no effect on rundown of currents evoked by kainate (20 μM).

Response for one neuron after 2 and 25 minutes of whole-cell recording in the presence of phalloidin are shown in the left panel. Kainate currents ran down at the same rate in the presence of phalloidin (right panel).

Cytochalasins induce rundown by antagonizing the action of ATP.

As reported by MacDonald et al. (1989), rundown of NMDA currents in whole cell recording could be prevented by including an ATP regenerating solution in the recording pipette (Fig. 3A, left panel). In the presence of ATP, the current at 25 minutes of recording was 102.6 ± 2.3 % (n=10) of that at 1-2 minutes. Filamentous actin is complexed with ATP and the hydrolysis of ATP to ADP by endogenous ATPase activity reduces the stability of the polymer (Sampath and Pollard, 1991). If actin depolymerization results in channel rundown, then high ATP concentrations in the regenerating solution could act by stabilizing actin filaments. To test this possibility, we examined the effect of cytochalasins on channel rundown. Cytochalasins enhance the hydrolysis of ATP-actin (Sampath and Pollard, 1991), and thus should overcome the action of the ATP regenerating solution. Consistent with our hypothesis, inclusion of cytochalasin D (1 μM) in the ATP regenerating solution induced current rundown. The response at 25 minutes was 76.6 ± 4.8 % (n=7, p=.001). Similar results were obtained with cytochalasin B (1 μM, 68.7 ± 1.9 %, n=8, p<.001) and cytochalasin B, dihydro (1 μM, 72.2 ± 2.4 %, n=5, p=.001). Cytochalasin B can also inhibit glucose transport (Cooper, 1987), however removing glucose from the extracellular solution had no effect on current amplitudes in the absence (n=4) as well as presence of 1 μM cytochalasin B (n=3). In addition cytochalasin D and cytochalasin B, dihydro have no effect on glucose transport.

Actin filaments are also destabilized by botulinum C2 toxin which ADP-ribosylates actin (Reuner et al., 1987). Thus we pretreated cultured hippocampal neurons with botulinum toxin C (250 ng/ml) for 18-30 hrs prior to whole-cell recording. Most neurons showed altered morphology with rounded somata,

however, seal formation and the stability of whole-cell recording remained unaltered. For the botulinum toxin-treated cells, NMDA responses in the presence of ATP regenerating solution ran down to 76.8 ± 5.7 %, (n=5), providing further evidence for the role of actin filaments in channel rundown. Microtubules are also present in dendrites (Black and Greene, 1982; Letourneau et al., 1987), thus we also examined reagents that specifically depolymerize or stabilize microtubules. Depolymerization of microtubules with colchicine (Black and Greene, 1982) did not induce rundown (Fig. 3C, right panel) With colchicine (10 μM) and ATP in the whole-cell pipette, the current at 25 minutes was 107.8 ± 3.1 % (n=4). This was not significantly different compared to ATP alone (Figure 3C, right panel). Intracellular taxol (5 μM), a cytotoxin that stabilizes microtubules in their polymerized form (Letourneau et al., 1987), did not prevent rundown. although the response after 25 minutes was slightly larger than rundown in control cells (70.4 ± 3.4 %, n=8, p = .01, Fig. 3C left panel). Thus rundown of NMDA channels appears to primarily involve the actin cytoskeleton.

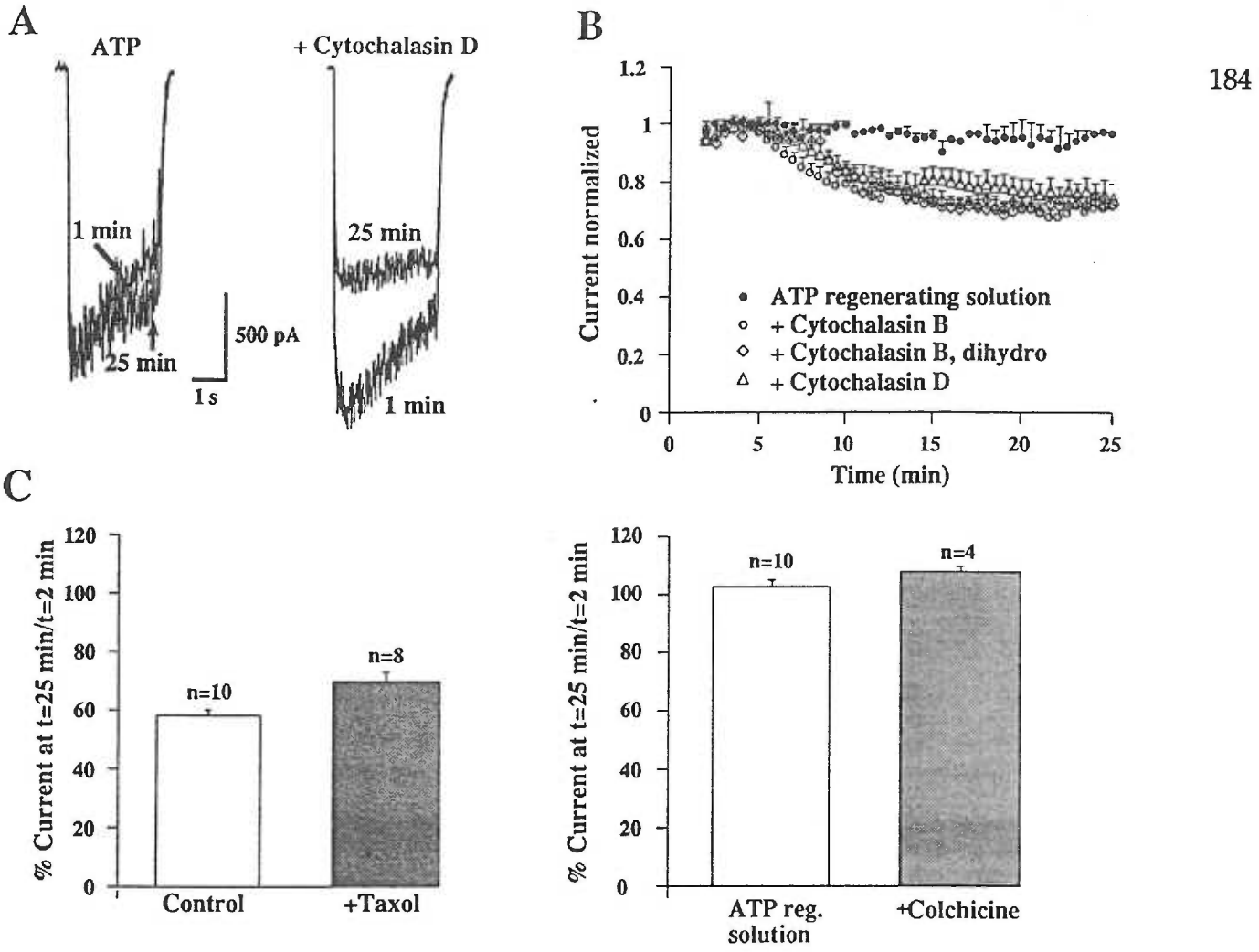


Figure 3. The prevention of rundown by ATP is overcome by cytochalasin.
A. Whole cell currents evoked by NMDA (10 μM) are shown for two neurons at 2 and 25 minutes of whole-cell recording in the presence and absence of cytochalasin D (1 μM). ATP regenerating solution was included in both cells. Cytochalasin D induced channel rundown.
B. ATP regenerating solution prevented channel rundown (closed circles). Cytochalasin B (open circle), cytochalasin D (open triangle) and cytochalasin B-dihydro (open diamond) each added to the pipette in a concentration of 1 μM resulted in gradual rundown of the current.
C. In the absence of ATP regenerating solution, the microtubule stabilizing agent, taxol (5 μM) did not prevent rundown. In the presence of ATP regenerating solution colchicine (10 μM) which depolymerizes microtubules, did not induce channel rundown. Control groups in the absence and presence of ATP regenerating solution (see Fig. 2A, 3B) are shown for comparison.

It has previously been suggested that ATP prevents NMDA channel rundown by phosphorylation of the receptor or an associated regulatory protein (MacDonald et al., 1989). Conceivably, the actin cytoskeleton could be important in the localizing or anchoring of kinases near the channel. We tested this possibility by adding kinase inhibitors to the pipette in the presence of phalloidin. However, staurosporine (1 μM) and calmidazolium (10 μM) did not overcome the action of phalloidin (99.4 ± 1.4 %; n=7).

Phalloidin does not block calcium-dependent inactivation

Either ATP regenerating solution or phalloidin were sufficient to prevent channel rundown. However, in the presence of ATP the channel still shows calcium-dependent inactivation (Legendre et al., 1993). As shown in Figure 4A, phalloidin also did not block calcium-dependent inactivation. During a 15 s pulse of NMDA (10 μM) the current decayed by 44.1 ± 3.8 % (n=6) in the presence of ATP regenerating solution. A similar degree of inactivation occurred in the presence of phalloidin (1 μM, 42.2 ± 4.5 %, n=7). In the absence of extracellular calcium, inactivation was nearly abolished (4.2 ± 4.0 %, n=4, Fig. 4A, right panel). This suggests that there are two actions of calcium on NMDA channel activity, only one of which directly involves the actin cytoskeleton.

This additional action of calcium was also apparent when the Na/Ca exchanger was inhibited. Block of the Na/Ca exchanger during the interval between agonist applications transiently inhibits NMDA currents even in the presence of ATP regenerating solution (Rosenmund et al., 1991, Soc.Neurosci. Abstr. 17, 957). We repeated this experiment by substituting 1 μM phalloidin for ATP to the whole-cell pipette. After 25-35 minutes of recording during which

current amplitudes remained stable, the Na/Ca exchanger was inhibited by reducing the extracellular [Na] to 42 mM in the interval between drug applications. This reduces the driving force of the exchanger and prevents the extrusion of $[Ca^{2+}]_i$ (Blaustein, 1988), and resulted in a transient reduction in the NMDA current (68.0 ± 6.3 %, n=4, Figure 4B). As was the case for ATP, this inhibition was not blocked by phalloidin. This provides further evidence for a secondary action of calcium in regulating NMDA channel activity.

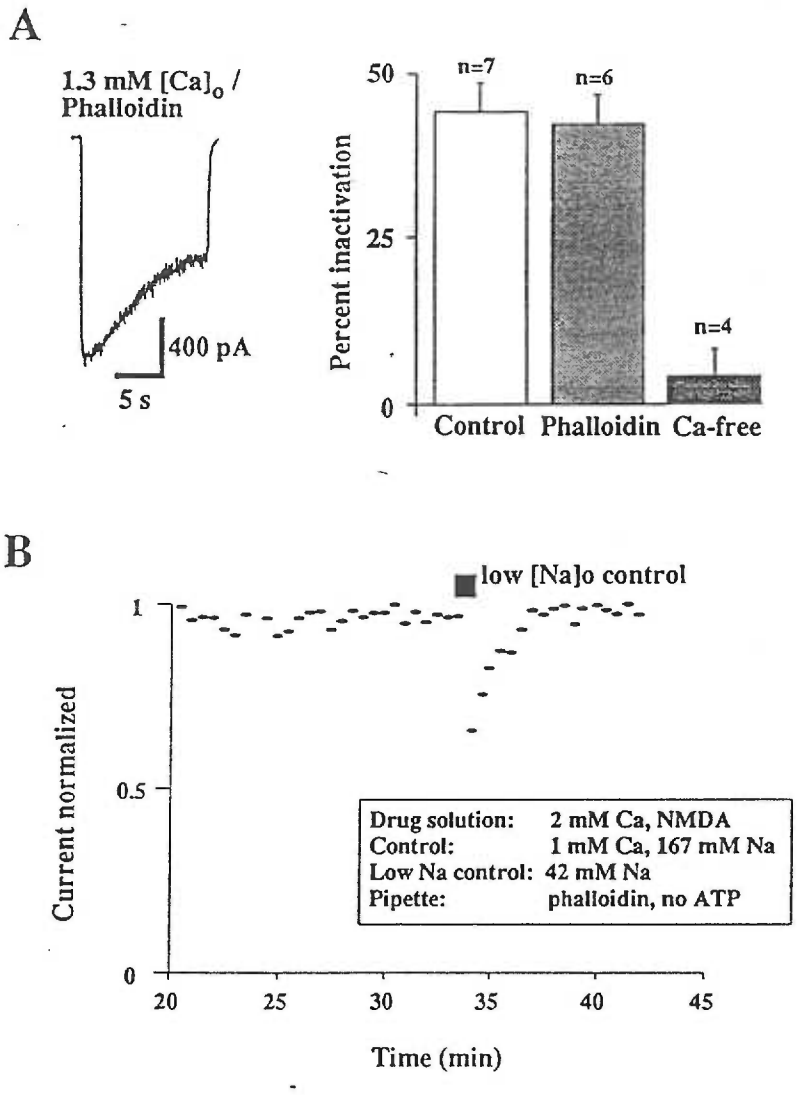


Figure 4. Phalloidin does not block the inhibition of the channel by intracellular calcium.

A. Response evoked in one neuron (left panel) by 15 second application of NMDA (10 μM) in 2 mM extracellular Ca^{2+}. The patch pipette contained phalloidin (1 μM), ATP regenerating solution and 10 mM EGTA. Phalloidin did not block calcium-dependent inactivation (right panel).

B. Phalloidin did not block the transient reduction of the NMDA current that occurred following inhibition of the Na/Ca exchanger. The Na/Ca exchanger was inhibited by lowering extracellular sodium to 42 mM in the interval between drug applications. The pipette contained phalloidin (1 μM) and no ATP.

Discussion

Regulation of NMDA channels by the actin cytoskeleton

Our results demonstrate that cytoskeletal interactions can regulate the function of NMDA channels in a calcium-dependent manner. We focused on actin as the probable target of NMDA-induced calcium transients because actin filaments are concentrated in the cortical cytoplasm of dendritic spines, and calcium depolymerizes actin filaments in vitro (Pollard and Cooper, 1986; Bamburg and Bernstein, 1991). We used two calcium-dependent changes in NMDA channel activity to assay the role of the cytoskeleton: calcium-dependent inactivation and channel rundown. Inactivation and rundown differ in that intracellular dialysis with ATP can prevent channel rundown, but not inactivation (Legendre et al., 1993). Stabilization of actin filaments with phalloidin mimicked the effect of ATP in preventing channel rundown whereas cytochalasins induced rundown in the presence of ATP. Thus ATP may stabilize NMDA currents by facilitating the assembly of actin filaments. In our experiments, rundown occurred with a time constant of several minutes. This was slower than the time constant of ATP-actin hydrolysis ($t_{1/2} \approx 2$ minutes in vitro, Pollard and Cooper, 1986). Thus the kinetics of actin depolymerization are sufficiently rapid to account for the observed rate of rundown. In addition, phalloidin and cytochalasins B-dihydro and D are highly specific reagents (Cooper, 1987), providing strong pharmacological evidence that calcium-dependence of rundown requires actin depolymerization. These reagents also did not alter AMPA/kainate receptors suggesting that NMDA channel rundown was not a result of nonspecific neuronal deterioration.

The calcium-sensitivity of NMDA channels depended on the patch recording configuration, and provides additional support for a role of the actin cytoskeleton. NMDA channels were calcium-insensitive in the inside-out configuration, suggesting that binding of intracellular calcium to the channel is not sufficient to account for the reduction in channel activity. Calcium and ATP also have little or no apparent effect on NMDA channels at steady state in outside-out patches (Sather et al., 1990; G. Tong and C. Jahr, personal comm.). However, we found that NMDA channel activity in cell-attached patches required 'gentle' seal formation (negative pressure = 1-5 mm Hg); under these conditions the activity was calcium-sensitive (Legendre et al., 1993). Gentle seal formation is also an important determinant of the activity of mechanosensitive channels (Hamill and McBride, 1992). Although excised patches can retain cytoplasmic components including cytoskeletal filaments (Sokabe and Sachs, 1990), the large negative pressures often used to form seals can lead to bleb formation (Milton and Caldwell, 1990). Membrane proteins in blebs show increased mobility (Tank et al., 1982), thus it it is likely that critical membrane-cytoskeletal interactions are disrupted. Taken together, these observations suggest that mechanical disturbance of the submembrane structure can affect NMDA channels,and that channel regulation by calcium requires an intact actin cytoskeleton.

Previous studies have linked glutamate-dependent calcium entry alterations in several cytoskeletal components. For example, glutamate can trigger the proteolytic destruction of fodrin by the calcium-dependent protease calpain I (Siman and Noszek, 1988). This has been suggested as a mechanism for upregulation of postsynaptic glutamate receptors (Lynch and Baudry, 1987).

Changes in the distribution of microtubule-associated proteins (MAPs) have also been reported following glutamate receptor activation possibly due to dephosphorylation of MAPs (Bigot and Hunt, 1990). However these mechanisms did not appear to be responsible for our results. First, nearly all experiments were performed in presence of protease inhibitors leupeptin or calpain inhibitor 1. In addition, disrupting microtubules with colchicine was ineffective and the microtubule stabilizing agent, taxol, had only a slight effect on channel rundown. Microtubules are virtually absent in postsynaptic spines (e.g. Landis and Reese, 1983), but they may be important for channels located in the cell body or dendritic shafts. For example, a 93 kD protein that copurifies with the glycine receptor has recently been shown to bind to microtubules (Kirsch et al., 1991).

Ion channels and the cytoskeleton

The traditional role of the actin-spectrin network in the cortical cytoplasm has been as a structural framework or anchor for various membrane proteins.This relationship has been best characterized in erythrocytes where the linker protein, ankyrin, attaches spectrin to the ion exchange protein; an interaction that is important to maintenance of the biconcave shape of the cell (Bennett, 1990). In axons, high densities of sodium channels are also concentrated at nodes of Ranvier by an interaction of actin, fodrin and and ankyrin (Srinivasan et al., 1988). Similarly, at the neuromuscular junction, the 43 kD protein, in association with spectrin and actin, appear to be responsible for clustering of AChR at release sites (Froehner, 1991). In the CNS, glutamate receptor are also concentrated at 'hot spots' (Trussell and Fischbach, 1989; Jones and Baughman, 1991), but the proteins responsible for this localization have not been identified.

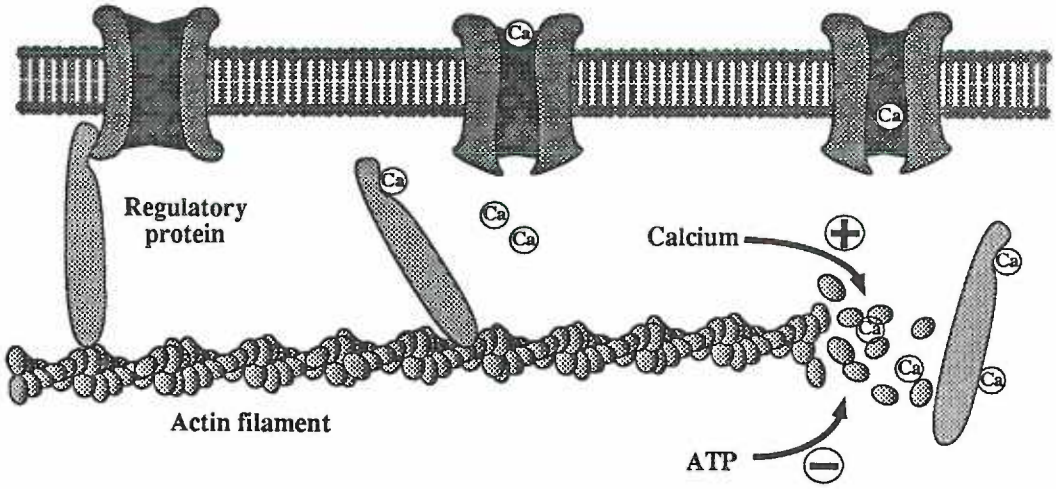
GABAA and glycine receptors have been reported to be immobilized on the soma and dendrites of cultured brain neurons (Velaquez et al., 1989; Srinivasan et al., 1990), presumably by a cytoskeletal interaction. In the case of the GABA receptor this does not appear to involve binding to ankyrin (Srinivasan et al., 1988). In addition, sodium channels bind to a 33 kD cytoskeletal protein whose function remains unknown (Edelstein et al., 1988). Thus there may be several classes of proteins linking receptors to the cytoskeleton.

Our results suggest a more dynamic role of the cytoskeleton in ion channel function. Such a mechanism has been postulated for mechanosensitive channels. Gating of "stretch-activated" channels is thought to rely on forces between the cytoskeleton and the attached membrane channels (Guharay and Sachs, 1984; Morris and Horn, 1991). Although the biochemical basis of this interaction is unexplored, disruption of actin by cytochalasin alters the behavior of MS channels (Sachs, 1986). This action is not calcium-dependent (Sachs, 1986; Hamill and McBride, 1992). However adaptation of the transduction current in vertebrate hair cells appears to involve a calcium- and actin-dependent mechanism. Calcium influx is thought to activate a molecular motor that maintains gating spring tension by moving along the actin core of the stereocilia (Shepherd et al., 1987; Hudspeth, 1989).

How does the actin cytoskeleton modulate NMDA channel activity?

A proposed model that incorporates our results is shown in Figure 5.

NORMAL INACTIVATED RUNDOWN



Three states of the channel can be distinguished from our experiments where both the inactivated and rundown state have an open probability that is approximately 50% of the normal activity (Legendre et al., 1993; Rosenmund, et al., Soc. Neurosci. Abst., 1991, 17, 957). Thus the receptor-cytoskeletal interaction is modulatory rather than an absolute requirement for channel opening. The transition to the rundown state, but not to the inactivated state, could be prevented by ATP or phalloidin. As both of these processes were calcium-dependent, it suggests that calcium has two actions. NMDA channels inactivate if calcium increases at the cytosolic face of the channel in intact cells, but not in in excised patches. Thus an additional component is necessary to maintain calcium sensitivity. This is represented in the model as a hypothetical regulatory protein that dissociates upon calcium binding. The channel can recover from inactivation as calcium is removed and the refulatory protein rebinds. Progression to the rundown state requires actin depolymerization that is mediated by calcium. Repeated calcium transients would be expected to destroy the structural integrity of actin network unless ATP (or phalloidin) is provided to allow actin repolymerization. This would eventually result in loss of the regulatory protein.

The model suggests that the regulatory protein is soluble and kept in place by direct binding to the actin cytoskeleton. However, as an alternative, the cytoskeleton could limit diffusion of membrane proteins in the submembrane compartment, or within the membrane. The existence of such 'corrals' has been proposed to explain the restricted diffusion of membrane proteins by actin-spectrin complexes in erythrocytes (Edidin et al., 1991). This could then facilitate an interaction between the channel and the regulatory subunit. We have not

specifically included spectrin/fodrin in the model, however calcium/calmodulin can regulate the binding of fodrin to actin (Sobue et al., 1983).

Although the model incorporates the available data, there is as yet no direct biochemical evidence of such a regulatory protein. However voltage-dependent channels have small molecular weight subunits that presumably act in a regulatory manner. For example, the β subunit of the calcium channel in skeletal muscle is thought to be associated with the intracellular surface of the channel complex (Catterall et al., 1988). The available glutamate receptor subunits have all been cloned by homology (Nakanishi, 1992), thus the existence of regulatory subunits has not been explored. As there are multiple acting-binding proteins, several of which are calcium-dependent (Bennett and Weeds, 1986), it is also possible that the regulatory subunit is one of the known actin-binding proteins.

Actin depolymerization and synaptic activity

Our results suggest that the ongoing contribution of NMDA channels to the postsynaptic response is a function of calcium homeostasis, energy supply and the structure of actin filaments. The effect of actin depolymerization on NMDA channels in dendritic spines has yet to be examined. However, in whole-cell recording this dynamic equilibrium can be shifted toward the inactivated or rundown state when the neuron experiences uncontrolled and/or long-lasting submembrane calcium transients. Cytoskeleton interactions could modulate the postsynaptic response on a rapid time scale, as occurs in cytoskeleton-mediated remodelling in growth cones (Smith, 1988).

Experimental Procedures

Cell culture

Cell cultures were prepared as described previously (Legendre and Westbrook, 1990).

Recording conditions

Experiments were performed in whole-cell or inside-out patch recordings on hippocampal neurons after 5-14 days in culture. Currents were recorded using an Axopatch 1C amplifier (Axon Instr.). Patch pipettes were fabricated from borosilicate glass (TWF 150, WPI) pulled with a conventional two-step puller (Sutter Instr.). Pipettes had 'bubble' numbers from 7.2 to 8.0. After fire polishing, the inner pipette tip diameter was 1.5 to 2.5 μM and the resistance ranged from 1-3 MΩ. Pipette solutions contained (mM): $CsMeSO_3$ or Csgluconate, 165; HEPES, 10; Cs_4-BAPTA, 2.4; $CaCl_2$, 0.4; $MgCl_2$, 2. The pH was 7.3 and the osmolarity 310 mOsm. The ATP regenerating system (Forscher and Oxford, 1985; MacDonald et al., 1989) including K_2-ATP, 4; K_2-creatine phosphate, 20; Phosphocreatine kinase, 50 U/ml and $MgCl_2$, 6; CsMeSO3 was reduced to 120 mM. In most experiments the nonspecific protease inhibitor leupeptin (100 μM) was included in the pipette. To offset junction potentials, a side well containing the patch solution was connected via an agar bridge to the bath. For whole-cell recording cell capacitance and series resistance (60-90%) were compensated.

Reagents were introduced into the cell by diffusion from the pipette. In some experiments, pipettes were backfilled, and the time between seal formation and membrane rupture was delayed for 3 minutes to reduce the exchange time during whole-cell recording. Only recordings with access resistance below 8 MΩ were included in the analysis. The extracellular medium contained (mM): NaCl, 167; KCl, 2.4; HEPES, 10; glucose, 10; glycine 0.01. NMDA was diluted in either 0 or 2 mM calcium while the control solution contained 1 mM calcium. The osmolarity was 325 mOsm and the pH 7.2. Tetrodotoxin (500 nM), picrotoxin (100 μM) and strychnine (2 μM) were added to inhibit spontaneous activity as well as ligand-gated chloride channels. Solutions were applied using a series of flow pipes (400 μM I.D.) positioned within 100-200 μM of the neuron, and connected to gravity fed reservoirs. Each flow pipe was controlled by solenoid a valve and the assembly was moved with a piezoelectric bimorph (Vernitron). For single channel recording in inside-out patches, NMDA (10-1000 μM) or glutamate (2μM) was added to the patch pipette along with 5 mM EDTA and EGTA. The gigaohm seal was made with large pipettes (2-3 μM inner tip diameter) and slight negative pressure (1-5 mm Hg), controlled by pressure transducer (Bio-Tek). Seal formation usually developed slowly (10-100 s) before excising the patch in Ca^{2+}-free solutions. Solution delivery was similar to whole-cell except that flow pipes were fabricated from square 4-barrel tubing with tip diameters of 100 μM. NMDA channels were identified based on their characteristic conductance levels (Jahr and Stevens, 1987), the reversal potential and by voltage dependence by Mg applied to the intracellular face of the membrane (Johnson and Ascher, 1990).

Experimental protocols and data analysis

Cells with leak currents > -80 pA at holding potential -60 mV were excluded to prevent nonspecific 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. Membrane currents were acquired using PClamp (V.5.5, Axon Instr) on an IBM-compatible computer and analyzed on a Macintosh using AXOGRAPH software (Axon Instru.). For analysis of rundown, NMDA (10 μM) or kainate (20 μM) was applied every 30 seconds for 3 seconds. To measure rundown, the peak response at 1-2 minutes was compared with the response after 25 minutes of whole-cell recording. For inside-out patches, the open probability and single channel conductance were measured by point-by-point amplitude histograms. The mean open time was calculated as the arithmetic mean of events detected using a 50 % threshold criteria. Data are expressed as percent of control ± SE. Significance was tested using Student's t-test.

Materials

Chemicals were obtained from the following sources (brackets indicate solvent used for stock solution, if other than H_20): Calbiochem: K_2-ATP, K_2-creatine phosphate; Molecular Probes: Cs_4-BAPTA; Sigma: phosphocreatine kinase/rabbit muscle, leupeptin, calpain inhibitor type 1, phalloidin, cytochalasin B, -D, -B-dihydro (dimethylsulfoxide, DMSO), colchicine, botulinum toxin C, calmidazolium (DMSO), staurosporine (ethanol, 70%). Taxol (ethanol, 70%) was a gift of Dr. Ver Narayanan (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, NCI, NIH).

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DISCUSSION

Effects of intracellular calcium on the NMDA channel

Earlier studies on the NMDA channel suggested that channel activity was inhibited by intracellular calcium (Mayer et al., 1987). However, several subsequent investigator reported that NMDA channel desensitization as well as rundown were calcium-insensitive (Clark et al., 1990; MacDonald et al., 1989). Our studies support the former proposal. Both phenomena require intracellular calcium.

Whole-cell NMDA currents inactivated as a result of elevated cytosolic calcium concentration. In addition, NMDA channel rundown required influx of calcium into the cytosol. Both inactivation and rundown were incomplete, reaching a maximum of approximately 50% of the peak response. in addition, inactivation was no longer apparent following rundown, suggesting that both processes have a similar effect on channel behavior. An examination of channel properties revealed a specific reduction of open probability during rundown. The channel conductance, mean open time, channel numbers and agonist affinity were unaffected.

Calcium acts via two mechanisms

Further studies on the mechanism of intracellular calcium revealed several differences between inactivation and rundown. The onset for maximal inactivation was less than 500 ms. In contrast, the development of rundown occurred over minutes, but the rate was highly dependent on the amount of calcium influx. Intracellular dialysis of ATP regenerating solution

in whole-cell recording blocked channel rundown, but not inactivation. Inactivation resisted any attempt of modulation, except methods that manipulated the effective cytosolic calcium concentration such as high concentration of exogenous buffer. Direct exposure of the channel to cytosolic calcium in inside-out patch recordings did not mimic inactivation, suggesting that an additional 'factor' for the effect of calcium was required, possibly a regulatory channel binding protein.

To account for rundown we have proposed a displacement of a regulatory protein held in place by the actin cytoskeleton, resulting in the loss of channel activity. Repetitive calcium transients result in the depolymerization of the actin cytoskeleton (Forscher, 1989). Consistent with such an model, rundown was blocked when depolymerization of the actin cytoskeleton was pharmacologically inhibited.

The action of ATP is on actin

Possible enzymatic mechanisms underlying the action of ATP in maintaining NMDA responses were studied.

Phosphorylation/dephosphorylation mechanisms were found to be only secondary, based on the narrow range of modulation exerted. The most significant effect occurred upon blockade of calmodulin. However the lack of effects of specific CaMKII and calcineurin inhibitors suggest that the protein regulated by calmodulin is not a calcium-calmodulin dependent kinase or phosphatase.

Calcium extrusion requires ATPase activity, thus the role of calcium pumps and exchanger in the maintenance of NMDA responses was

examined. The Na/Ca exchanger was found to have an important role in controlling calcium homeostasis. The nonspecific P-ATPase inhibitor vanadate also effectively inhibited the effect of the ATP regenerating solution, consistent with a participating role of ATP in the calcium extrusion.

The most significant effects of ATP was attributed to the actin cytoskeleton. Actin filaments (F-actin) continuously hydrolyze complexed ATP, and depletion of ATP will lead to a unstable polymer (Forscher, 1989). Thus removal of ATP via whole-cell dialysis or by enhancing hydrolysis with reagents such as cytochalasin, forces actin into the monomeric (G-actin) state. Consistently the three cytochalasins tested induced channel rundown. Phalloidin, a fungal toxin that blocks actin depolymerization, blocked rundown in absence of ATP. However, neither ATP not phalloidin interfered with calcium-dependent inactivation. These two common features of ATP and phalloidin on channel activity suggests that ATP maintains NMDA responses primarily by an action on actin cytoskeleton.

Figure 5 of the third manuscript outlines our working hypothesis. Calcium entering through the NMDA channel binds to a calcium binding site at the NMDA channel/protein complex and causes dissociation of the regulatory protein. The channel open probability is subsequently reduced by up to 50 %, resulting in calcium-dependent inactivation. Upon removal of calcium, reassociation occurs. However, destruction of the cortical cytoskeletal network under rundown conditions causes irreversible loss of the subunit.

Does rundown in whole-cell recordings reflects an physiological process?

Although our experiments did not directly demonstrate actin-dependent rundown of NMDA channel activity under physiological conditions, several facts support the physiological relevance of our findings. High turnover rates of actin, for example in growth cones, have been demonstrated (Smith, 1988). Second, calcium is a potent mediator of actin turnover *in vitro* (Pollard and Cooper, 1986). Third, actin is a major protein the postsynaptic spine (Matus et al., 1982). Forth, changes in NMDA responsiveness in our experiments (manuscript II) occurs on large time scales such as in LTD (Artola et al., 1992). Fifth, whole-cell dialysis in our experiments interfered with energy depletion and calcium homeostasis, but did not cause rundown per se (manuscript II).

Under physiological conditions, the proposed loss of the regulatory protein during rundown of whole-cell recordings could be envisioned to be an remodeling of channel/protein complexes following larger calcium transients. The temporary softening of the cortical cytoskeleton would permit that new channel/protein interactions could be formed, substituted or eliminated, resulting in potential changes in the channel activity.

Future directions

Although the proposed model incorporates the available data on NMDA channel regulation by calcium, several steps in the mechanism of calcium, in particular with respect to inactivation remain to be determined.

* What is the nature of the regulatory protein?
* Where is the calcium receptor?
* Are subsynaptic NMDA channel calcium-sensitive?

The first two questions clearly require biochemical and molecular biological methods. The subunit could be isolated using fragments of cloned NMDA subunits (Nakanishi, 1992), in particular cytosolic, that could serve as probes or ligands for the subunit. As the subunit may have not sequence homology to NMDA channel clones, a rational approach could be the isolation of the protein using the protein-protein interaction of the subunit to sequences of NMDA clones. Unless the subunit is an ubiquitous protein, the interaction of the subunit with the channel should be of relative high specificity. The bimolecular interaction should also be sensitive to calcium.

The cloned NMDA channel subunits NMDAR-2a and NMDAR-2b, but not NMDAR-2c have an interesting structure in the putative cytosolic loop between the transmembrane-spanning regions 3 and 4. A zinc-finger-like structure, that could have divalent binding properties (Monyer et al., 1992). This hypothesis is supported by the observation that the NMDAR clones 2a and 2 have differential sensitivity to ifenprodil (Williams et al., personal

communication). The action of ifenprodil shares a similar range of modulation with calcium (Legendre and Westbrook, 1991).

The simplest way to test this hypothesis would be to measure Ca-dependent inactivation in cell lines that specifically express NMDAR-2a,b or NMDAR-2c subunits. However, it is possible that the cell lines do not express the required 'non-channel' subunit. Thus, such experiments may require the isolation of the subunit from native tissue. As potential subunits are isolated, there physiological function could be tested by reconstituting patches containing NMDA channel activity and testing for calcium sensitivity. As recent studies suggest a developmental regulation of NMDA channel function, it would be interesting to look at the regulation of developmental expression of these subunits (Hestrin, 1992).

The third question whether NMDA channels in the synapse are inactivated by calcium is important to show the physiological relevance of the observed phenomena, and could provide a potential mechanism of LTD (see page 8). This could be addressed with several approaches. The effects of intracellular cytochalasins or phalloidin could detect a cytoskeletal component in the postsynaptic behavior of the NMDA channel. Paired-pulses of NMDA synaptic currents could be measured in different calcium concentrations. As presynaptic release is sensitive to calcium, the AMPA component of the synaptic response could be used to normalize the change in synaptic amplitude to the calcium sensitive component. However the maximal amount of inactivation is dependent on the presynaptic release probability, determining the ratio of synapses that are activated during both

pulses. A more direct way of elevating postsynaptic spine calcium is by the photolytic release of 'caged' calcium (Tsien and Zucker, 1986).

Significance

Our findings may contribute to two specific areas of neuroscience research. First, the mechanism of NMDA channel regulation identified here introduces a new principle that involves the cortical cytoskeleton. It may stimulate the search for cytosolic proteins that regulate NMDA channel gating, presumably such regulatory subunits will not be identified by sequence homology to cloned NMDA channel subunits.

Second, our findings may be an example for more general principles that organize the function of submembrane compartment of excitable cells. The type of manipulations performed in patch clamp techniques reveal disintegration of the function of membrane proteins, measured in the altered behavior or rundown of many channels. We may still underestimate the functional and structural entity of the membrane/submembrane compartment as an cellular organ. As a metaphor, the skin, our largest organ and essential in the communication with the outer world, is often seen solely as a physical border.

Conclusion

NMDA channels in cultured hippocampal neurons of rat are regulated by intracellular calcium. Although the two characterized phenomena - inactivation and rundown - led to the same inhibition of the channel open probability, the underlying mechanism were distinct. Rundown appears as a calcium-induced actin depolymerization, however the mechanism of inactivation may involve the interaction of the NMDA channel with a yet unidentified cytosolic protein. Inhibition of NMDA channels by calcium may be clinically important, as uncontrolled increases in intracellular calcium are hypothesized to play a critical role in a number of neurological diseases. Calcium-dependent inactivation may serve as a feedback signal preventing the excessive calcium accumulation in the neuron.

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