Electrophysiology and plasticity in isolated postsynaptic densities

Ursula Wyneken a,*, Juan José Marengo b, Fernando Orrego a

a Laboratorio de Neurociencias, Facultad de Medicina, Universidad de los Andes, San Carlos de Apoquindo 2200, Las Condes, Santiago 6782468, Chile
b Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile and Institute of Neurosurgery Prof. Asenjo, Chile

Abstract

The organization and regulation of excitatory synapses in the mammalian CNS entails complex molecular and cellular processes. In the postsynaptic membrane, scaffolding proteins bring together glutamate receptors with multiple regulatory proteins involved in signal transduction. This gives rise to an elaborate postsynaptic structure known as the postsynaptic density (PSD). This protein network plays a critical role in the regulation of glutamate receptor function and thus in synaptic plasticity. To study this regulation, we have developed a system in which ionotropic glutamate receptors (iGluRs) can be recorded, in the steady state, by the patch clamp technique in isolated PSDs incorporated into giant liposomes. In this preparation, ionotropic glutamate receptors maintain their characteristic physiological and pharmacological properties. The recordings reflect the presence of channel clusters, as multiple conductance and subconductance states are observed. Each of the receptor subtypes is activated by a specific set of kinases that are activated differentially by Ca²⁺: the “kainate receptor kinases” are active even in the presence of EGTA, i.e. they are not calcium-dependent; the “N-methyl-D-aspartate receptor (NMDAR) channel kinases” are active in the presence of submicromolar calcium concentrations, whereas the “α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptor kinases” need μM calcium for activation. The NMDA receptor showed its characteristic voltage-dependent Mg²⁺ blockade, and activation by phosphorylation was in part a consequence of a relief of Mg²⁺ blockade. These results allow us to propose a model in which phosphorylation of NMDA receptors can contribute to a long-lasting and self-maintained change in synaptic function.

The experimental approach we present will allow us to test the functional consequence of activation of the multiple signal transduction pathways thought to regulate excitatory neurotransmission in the adult CNS.

Theme: Excitable membranes and synaptic transmission
Topic: Postsynaptic mechanisms

Keywords: Glutamate receptors; Postsynaptic density; NMDA; Phosphorylation; Plasticity

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E-mail address: uwyneken@uandes.cl (U. Wyneken).
1. Introduction

In this review, we show the advantages of studying glutamate receptor function in the postsynaptic density (PSD), the structure where they are specifically anchored in the CNS.

Rapid excitatory neurotransmission is mediated mainly by ionotropic glutamate receptors (iGluRs) which play important roles in synaptic plasticity, neuronal development and neurological disorders [9,24,25,77,106]. Pharmacological and molecular techniques have been used to identify three categories of iGluRs according to their agonist selectivity and sequence homology: N-methyl-D-aspartate (NMDA)-type receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)-type receptors and kainate receptors [38,85]. iGluR subunits have been cloned and their cDNAs expressed [107,121]. To date, three major classes of NMDA receptor subunits (NR1, NR2A-D and NR3A-B), four AMPA receptor subunits (GluR1-4), and five kainate receptor subunits (GluR5-7 and KA1-2) have been reported [38]. All three families of glutamate receptors can co-assemble within families, possibly as tetramers [103,76], to produce many receptor combinations [4,135]. Alternative splicing and RNA editing generate further variability contributing to a great molecular and functional diversity [24,76].

The subunit composition of NMDARs determines many aspects of their physiology [121]. Conventional heteromeric NMDARs, which contain NR1 and NR2 subunits, require dual agonists for activation: glutamate that binds to NR2 subunits and glycine or D-serine that bind to NR1 subunits [46,87]. Remarkably, coexpression of NR3 with NR1 and NR2 subunits suppresses glutamate-induced currents. NR3A or NR3B co-assemble with NR1 when co-expressed in Xenopus oocytes to form excitatory glycine receptors that are unaffected by glutamate or NMDA and inhibited by D-serine [15]. In addition, NMDARs display sensitivity to different endogenous modulators: they are stimulated by Zn²⁺ and polyamines [83].

AMPA and kainate receptors, that assemble as homo- or hetero-oligomers, mediate rapid synaptic transmission at the postsynaptic level. The contribution of kainate receptors to this phenomenon has been more difficult to assess [60,62,96,97]. In addition to the well-established role of AMPARs in synaptic plasticity [74,78], kainate receptors can regulate neurotransmitter release at excitatory and inhibitory synapses and therefore have a role in plasticity, too [41,60].

Regulation of postsynaptic glutamate receptors is one of the main mechanisms for altering synaptic efficacy in the central nervous system. One of the most important mechanisms involved is phosphorylation and dephosphorylation [123]. The regulation of GluR channels by kinases and phosphatases plays an essential role in changes of synaptic strength, such as long-term potentiation (LTP) and long-term depression (LTD) [63,77,115]. The fundamental role of NMDARs in the induction of LTP is well established. NMDARs are highly permeable to Ca²⁺ and exhibit voltage-dependent inhibition by extracellular Mg²⁺ [81,92]. At depolarizing membrane potentials, when Mg²⁺ block is relieved, Ca²⁺ enters the cell and triggers intracellular biochemical cascades that produce long-lasting changes in neuronal function. A simplified model is that Ca²⁺ entry ultimately leads to an increase in AMPAR-mediated current and thus results in a potentiation of synaptic efficacy. One of these biochemical events is activation of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), that is essential in LTP and behavioural memory [73,114]. However, also important roles have been assigned to the combined activation of the src family of tyrosine kinases [3], protein kinase A (PKA) [5] and protein kinase C (PKC) [75]. The consequence of GluR phosphorylation is an enhancement of glutamate-activated currents [115]. In addition, crosstalk between these and other complex biochemical pathways exists, and the dephosphorylation of synaptic currents has been shown to be mediated by protein phosphatases that can dephosphorylate iGluRs [59,66,145].

1.1. Regulation by phosphorylation of iGluRs

The study of the glutamate-gated ion channels has been dominated by the molecular identification of the receptor isoforms and their biophysical characterization [21]. Phosphorylation/dephosphorylation studies have mostly been done by expression in heterologous systems in vitro or in neurones in culture, and varying or even contradictory results have been found. One of the most illustrative examples are the studies regarding NMDAR regulation by PKC, which are summarized in Table 1. NMDARs are potentiated by PKC in trigeminal neurons which reduces its voltage-dependent Mg²⁺-block [16]. PKC phosphorylates primarily but not exclusively the C1 cassette of the...
NMDA subunit NR1, both in neurones in culture and in heterologous expression systems [125]. However, recombinant receptors lacking the C1 cassette exhibit greater phorbol-ester induced potentiation than do those containing this cassette, when studied in oocytes expressing different splice variants of the NR1 subunit [27]. The effect of PKC activation is NMDAR inhibition when studied in cerebellar granular cells in culture [20,112]. Also, in CA1 pyramidal neurons in culture, the steady-state NMDAR-mediated current is depressed in a calcium-dependent manner, whereas the peak current is enhanced [72]. However, when expressed in oocytes, NMDARs are activated by PKC in a calcium-dependent manner [111,149,151]. This is probably mediated by a PKC-induced membrane insertion and activation of NMDARs that is also seen in embryonic hippocampal neurons in culture [57]. A PKC- and src-dependent membrane insertion of NMDARs in hippocampal slices of adult brain has been reported [34]. Recently, it has been shown that PKC induces dispersal of NMDARs in an heterologous expression system [126] and in rat hippocampal cultures, wherethey move from a synaptic to an extrasynaptic localization [29]. The conflicting results may

Table 1

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<th>Biological system</th>
<th>Subunit</th>
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<tr>
<td>(I) Stimulatory effect</td>
<td>Oocytes</td>
<td>NR1 splice variants</td>
<td>degree depending on splice variant</td>
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<td>NR1 splice variants NR2A</td>
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<td>Oocytes</td>
<td>NR1(_{100}/NR2A)</td>
<td>potentiated by intracellular Ca(^{2+}) with PSD-95</td>
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<td>Oocytes</td>
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<td>channel gating no effect when coexpressed</td>
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<td>Oocytes</td>
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<td>mediated by the NR2B tail phosphorylation no effect on Mg(^{2+}) block</td>
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<td>NR1/NR2A to D (mouse homologs)</td>
<td>inhibits rundown no effect on rundown</td>
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<td>HEK 293</td>
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<td>reduces Mg(^{2+}) block</td>
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<td>HEK 293</td>
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<td>Trigeminal isolated neurons</td>
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<td>nd</td>
<td>via src</td>
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<td>Hippocampal isolated neurons neonatal/12–20 d)</td>
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<td>of peak current via AK(_{h}/)src</td>
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<td>no effect on Mg block Ca(^{2+}) independent</td>
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<td>(II) Inhibitory effect</td>
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<td>of steady-state current by enhancement of Ca(^{2+})-dependent inactivation</td>
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<td>Hippocampal slices (adult)</td>
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<td>Oocytes</td>
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<td>Fibroblasts</td>
<td>NR1</td>
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CAK\(_{h}\): cell-adhesion kinase \(\beta\)/proline-rich kinase 2 (Pyk2), d = days, nd = not detected. NR1 splice variant nomenclature (Durand, 1993): The eight possible splice variants, seven of which are expressed in the CNS, arise from splicing in or out three exons in the N-terminal domain (N1), and in the C-terminal domain (C1 and C2). Splicing out of the second C-terminal domain deletes a stop codon and results in an additional open reading frame before arriving at a new stop codon. NR1 variants are denoted by the presence (1) or absence (0) of the three alternative spliced exons, for example the splice variant NR1011 corresponds to the following exon arrangement: N1 absent, C1 present, C2 present.
be in part due to the fact that Fong et al [29] studied synaptic versus extrasynaptic receptors, whereas the other authors made no distinctions between them. Supporting the idea that synaptic receptors might behave differently relative to extrasynaptic ones, Yamada et al. [143] found that the PKC potentiation of NMDARs expressed in oocytes disappeared when coexpressed with PSD-95, the synaptic NMDAR scaffolding protein. The different biological systems used in the PKC studies may contribute to different results, whereas the consequences of PKC activation on exclusively synaptic NMDAR functioning have not been addressed. It is well known that kinases can phosphorylate many proteins in various systems, although only some of them are their real physiological substrates.

The role of tyrosine kinases and of other serine–threonine kinases has been less controversial. For example, the NR2B subunit of NMDARs is phosphorylated on tyrosine residues in isolated postsynaptic densities [86], in hippocampal neurons [58] and in heterologous expression systems [124,146]. Tyrosine phosphorylation, mediated by the src family of kinases, produces NMDAR upregulation in cultured neurons [3,147]. Additionally, the upregulation of NMDARs in hippocampal neurons by PKC or metabotropic receptors is mediated by src tyrosine kinases [71]. One of the current models for NMDAR upregulation by src family of kinases has become even more complex: PKC activates the cell-adhesion kinase β (CAKβ) that in turn binds to and activates src [40]. At least one paradox remains to be resolved: src-induced potentiation of recombinant NR1/NR2A receptors in HEK293 cells is due to a reduction of channel inhibition by extracellular Zn2+ [150] while this mechanism does not operate in neurons [142]. The precise role and fine regulation of phosphotyrosine content in NR2B at the synapse is not yet known, although there is a general agreement that src upregulation of NMDARs may be important in many physiological processes, like LTP [84,102,104].

A CaMKII phosphorylation site has been identified in the NR2B subunit, but no regulatory function has been reported [93]. Although CaMKII associates with NMDARs [32,68], it phosphorylates primarily AMPAR GluR1 subunits [6,79], causing an increase in single channel conductance when expressed in an heterologous system [22]. GluR1 phosphorylation by PKA controls the AMPAR open probability in HEK 293 cells [5] and LTP in hippocampal slices is associated with increased phosphorylation of CaMKII and PKA consensus sites of GluR1, whereas their dephosphorylation correlates with LTD [59], suggesting a role for PKA in the physiological regulation of AMPARs. GluR4 is phosphorylated by PKA, PKC and CaMKII in vitro [12,13,18]. The incorporation of AMPAR subunits into synapses is regulated by PKA phosphorylation of GluR4, whereas GluR1 incorporation requires activation of both PKA and CaMKII [28].

Accumulating evidence demonstrates that the GluR phosphorylation status is dynamically controlled in vivo by phosphorylation/dephosphorylation cycles, and that inhibition of protein phosphatases potentiates GluR function [8,54,59,66,113,134,145].

1.2. Synaptic iGluRs are anchored to PSDs

Excitatory synapses in the mammalian brain occur mostly on dendritic spines, where receptors and downstream signalling enzymes are clustered in the PSD. The PSD is a cytoskeletal web beneath the plasma membrane that contains five classes of proteins: neurotransmitter receptors, cell-adhesion proteins, adaptor molecules, signalling enzymes and cytoskeletal proteins [47]. Many of them have recently been identified by mass spectrometry [42,133]. In this complex network, specific signalling modules are brought together by scaffolding proteins that contain PDZ domains [127]. The prototypical PDZ protein, PSD-95/SAP90, belongs to the family of the membrane-associated guanylate kinases (MAGUKs), comprised of four closely related proteins. MAGUK proteins contain five domains involved in protein–protein interactions: three amino-terminal PDZ domains followed by an SH3 domain and a GuK domain homologous to yeast guanilate kinase but lacking enzymatic activity. The first and second PDZ domains bind tightly to the carboxy-terminal tails of the NR2 subunits of the NMDAR [55,90]. The third PDZ domain interacts with CRIP, one of the probable mechanisms that link the NMDAR to the cytoskeleton [91,95]. Other proteins that interact with PSD-95 are the neuronal nitric oxide synthase [10], the kainate receptor subunits KA2 and GluR6 [31], the neuronal Rho-GEF Kalirin-7 [98], neurelins [43], SynGAP, a synaptic ras GTPase-activating protein [51], GKAP that interacts with the GK domain [49], Shaker-type potassium channels [48], a Ca2+-ATPase isofrom [50] and ephrins and their receptors [129].

In addition, a lattice of scaffold proteins can link metatropic GluRs to the NMDAR complex [130], whereas AMPARs form part of a different signalling complex. While immature AMPARs associate with SAP97, a member of the MAGUK family [105], mature AMPARs are anchored to the PSD by another type of PDZ domain-containing proteins called glutamate-receptor-interacting protein (GRIP) [26], ABP [118] and Protein interacting with C kinase (PICK1), which targets PKCo to AMPARs [139].

The C-terminus of GluRs also interact with non-scaffold proteins. For example, AMPARs associate with the N-ethylmaleimide-sensitive factor (NSF) [117], whereas the NR2 subunit of NMDARs binds calmodulin and alpha-actinin [138], as well as CalMII, the most abundant signalling protein in the PSD. The NR1 subunit of the NMDAR can be complexed with protein phosphatase-1 and cAMP-depent protein kinase (PKA) by means of the scaffold protein Yotiao, while the protein phosphatase 2A associates with the NR3A subunit [14]. The presence of protein phosphatases 1 and 2A in PSDs may be involved in the temporally and spatially precise control of the phosphorylation state of its proteins [119,152].
As shown by electron microscopy of immunogold-labeled synapses, the NMDAR signalling complex forms the core of the PSD, whereas AMPARs are distributed more uniformly and metabotropic glutamate receptors are concentrated in the outer rim of the PSD [47,116]. This ordered PSD architecture is a consequence of association of iGluRs to specific proteins and hence distinct signalling systems, within the PSD. For example, synaptic NMDARs have anti-apoptotic activity mediated by CREB phosphorylation, whereas stimulation of extrasynaptic NMDARs triggers cell death [37]. Interestingly, extrasynaptic NMDARs can mediate inhibition by coupling to Ca²⁺-activated K⁺ channels [44].

1.3. Functional studies of iGluRs in PSDs

Although it seems that the molecular composition of synaptic iGluRs does not vary from extrasynaptic ones [2], many studies suggest that the functional properties of GluRs are modified when they are associated with the PSD or with their scaffold proteins. For example, clustering of kainate receptors by PSD-95 reduces its desensitization [31]. The association of NMDARs to PSD-95 decreases the sensitivity of the channel to L-glutamate and inhibits the PKC potentiation of the channels when expressed in oocytes [143], while coexpression of NMDARs and PSD-95 with GSK3β potentiates channel activity [144]. Liao [64] confirmed that PKC potentiation in oocytes was eliminated by PSD-95 coexpression, while src potentiation was promoted by PSD-95 coexpression in heterologous cells [64,124]. Phosphorylation on specific sites of GluR subunits depends on their PSD localization [122]. For example, although the AMPAR subunit GluR1 can be phosphorylated by PKA on Ser-845 in heterologous cells and in neurons [101], this site is mostly occluded in PSD-anchored receptors [131], so, it is difficult to assign a physiological role to it. Similar results were found regarding potential phosphorylation sites for src in PSD-anchored NMDARs [17].

Some attempts have been made to study iGluR function in isolated PSDs: NMDAR activity and its regulation by kinases was inferred studying the binding of the open-channel blocker MK 801 to them [52]. The regulation of iGluR sensitivity to agonists and antagonists has also been studied in isolated PSDs by measuring the binding of selective substances to each receptor subtype [7,108,128]. However, these attempts do not clarify many of the complex mechanisms thought to participate in iGluR regulation.

Based on these evidences, we propose that the function of iGluRs is specifically modulated by their anchoring in the PSD that, in turn, depends on the influence of other neighboring regulatory proteins, that are probably not present in extrasynaptic glutamate receptors, which are the ones usually subject to electrophysiological recordings. For this reason, we have developed a system, in which isolated PSDs can be recorded by the patch clamp method following their incorporation into giant liposomes. This has allowed us to record specific ionotropic glutamate receptor subtypes present in them, as well as their regulation by different modifications induced on the “intracellular” side of the PSD, where kinases and phosphatases associate in the vicinity of their targets.

2. Methods

2.1. Subcellular fractionation

For PSD isolation, the method of Carlin et al. [11] was slightly modified [137].

2.2. Electrophysiological recordings

Giant liposomes containing PDSs were prepared as described earlier [99]. Reconstituted glutamate receptors present in the PSD were activated by injection of 0.5 to 1 μl of the 10-fold concentrated agonist or antagonist into the recording pipette through a plastic catheter. The agonist concentrations in the injection solutions were: 50 μM NMDA (RBI) and 5 μM glycine for NMDARs, 5 μM glutamate-free quisqualate (Tocris) for AMPARs or 10 μM kainate for kainate receptors. The antagonists used were d-2-amino-5-phosphonovaleric acid (APV) for NMDARs and 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX) for non-NMDARs. A simplified scheme of the PSD isolation followed by incorporation of them into giant liposomes and their subsequent recording is shown in Fig. 1. Once the excised patch containing a PSD was obtained, a control recording was performed at the holding potential before injecting the agonist, in this case NMDA and glycine, which evoked a current specifically related to the injection. Patch clamp recordings were obtained using an EPC-9 patch amplifier (HEKA Electronics) at a gain of 50 mV/pA. Data analysis, like mean current values and current amplitude histograms of 1-min current traces, was performed using the Microcal Origin software. All measurements were performed at room temperature. All reagents except those specified were from Sigma.

3. Results

3.1. Presence of functional glutamate receptors in reconstituted PSDs

Membrane patches in which currents were present in the absence of glutamate or other glutamate receptor agonists in the patch pipette were discarded [99]. In silent seals, when glutamate was added to the “intracellular” face (i.e. to the bath) of a reconstituted PSD, no currents were evoked. However, when glutamate, or one of the agonists selective for each of the glutamate receptor subtypes, was injected into the patch pipette, i.e. close to the “extracellular” side of
the PSD, robust currents of a complex type were induced following a latency of 10–20 s, interpreted as due to diffusion of the agonist from the tip of the injection cannula to the PSD.

Kainate, at a 0.2–2 µM final concentration in the pipette, which is selective for activating kainate receptors, induced a complex current pattern (Fig. 2, top record), which is thought to represent the asynchronous openings and closures of multiple glutamate receptor channels clustered in the PSD. Similar patterns were seen when 1 µM quisqualate, an agonist of the AMPAR was injected together with 2 µM APV, an NMDA-receptor antagonist. These currents were blocked by the AMPA-kainate receptor antagonist, CNQX (Fig. 2, lower trace).

Activation of NMDARs by injection of 10 µM NMDA plus 1 µM glycine, in the presence of 2 mM “extracellular” magnesium (Mg²⁺)o, led also to very complex evoked currents (Fig. 3A) that were of much greater magnitude at positive “intracellular” potentials relative to those seen at negative ones (Fig. 3B). This indicated that the NMDARs present in this preparation also showed the well-established voltage-dependent Mg²⁺ block. Due to the rapid flickering of the receptors, the current amplitude histograms did not allow the identification of the multiple conductances that could be recognized visually (Fig. 3C), nor the calculation of the open probability or single conductance levels as activity index. For this reason, to assess channel activity, we have calculated the mean current flow in 1-min recordings. The fractional current flow at positive and negative potentials was calculated at each membrane potential by dividing the mean 1-min current at negative potentials by the mean 1-min current at positive potentials. In this case, the fractional current flow was of 0.246.
suggesting that the parameters that describe Mg\(^2+\) block seen before and after the addition of ATP–Mg\(^2+\) (Fig. 5).

membrane potential (injection of CNQX into the patch pipette. Both injections were done at a current on a previously silent seal. Lower trace: currents recorded in the injection of kainic acid to a final concentration of about 1 \(\mu\)M. Upper trace: currents recorded in the injection of CNQX into the patch pipette. Both injections were done at a membrane potential of +40 mV.

**Fig. 2.** GluR-mediated currents were activated or inhibited by the injection of specific agonists or antagonists into the patch pipette. Upper trace: injection of kainic acid to a final concentration of about 1 \(\mu\)M induced a current on a previously silent seal. Lower trace: currents recorded in the injection of CNQX into the patch pipette. Both injections were done at a membrane potential of +40 mV.

### 3.2. Effects of glutamate receptor phosphorylation

Addition of 2 mM ATP–Mg\(^2+\) to the “intracellular” face of the PSDs, a procedure which is known to phosphorylate a large number of PSD proteins by means of endogenous protein kinases present in the PSD, led to increased currents in kainate, AMPA and NMDA receptor activity (Figs. 4, 6 and 7). In the latter case, the results are of particular interest because phosphorylation, in the presence of 2 mM (Mg\(^2+\))o, resulted in large increases in currents seen both at negative and positive potentials (Fig. 4). In this set of experiments, the increases were of 153 ± 8% (n = 8, p < 0.01), and 103 ± 12% (n = 8, p < 0.01), respectively. The voltage-dependent current block seen in the presence of (Mg\(^2+\))o was markedly attenuated by phosphorylation, and the extent of this unblocking depended on the magnitude of the negative potential. A Boltzmann fit [19] of the relative currents \(I_i/I_s\) seen before and after the addition of ATP–Mg\(^2+\) (Fig. 5) showed that phosphorylation shifted the potential that gave a 50% current block, from ∼53.9 mV in control PSDs, to −74.8 mV, indicating that voltage-dependent Mg\(^2+\) block was greatly attenuated. Also, the parameter \(\delta\), that represents a measure of the electrical field distance that is crossed by Mg\(^2+\) to its blocking site, changed from 0.27 to 0.51, suggesting that the parameters that describe Mg\(^2+\) block change after phosphorylation, possibly because of a conformational change of the receptor. As in Fig. 5, the current ratio between positive and negative potentials is given; it could be argued that this ratio is enhanced at more negative potentials, because “intracellular”Mg (Mg\(_{\text{in}}^2+\)) could inhibit NMDA receptor currents at the corresponding symmetric positive potential [69]. We think this possibility is unlikely because phosphorylation increases the absolute magnitude of the current seen at negative potentials (cf. Fig. 4), and also because in an intracellular solution that contains 2 mM ATP, 2 mM Mg\(^2+\) and 0.1 mM EDTA at pH 7.4, the estimated concentration of free Mg ion is only 0.3 mM, a concentration that has no significant blocking effect on NMDA receptor currents at positive potentials [69].

3.3. Selective effect of different protein kinases

Phosphorylation of PSDs, in the presence of 0.1 mM EGTA (pCa 9), enhanced in them the current induced by 1 \(\mu\)M kainate by 157 ± 9% (Fig. 6). No further activation was found following the addition of 10 \(\mu\)M Ca\(^{2+}\) and 12.5 \(\mu\)g/ml of calmodulin, thus showing that the kinase responsible for this effect was calcium-independent.

In the case of AMPARs, the effect of phosphorylation was abolished when it was performed at pCa 9, and was maximal when 10 \(\mu\)M Ca\(^{2+}\) and 12.5 \(\mu\)g/ml of calmodulin were present (Fig. 6), as is to be expected due to the well-established functional association between CaMKinase II and this receptor [67,68].

NMDARs, on the other hand, showed a more complex pattern. Thus, 0.1 mM EGTA inhibited significantly the induced receptor currents, but the effect of ATP–Mg was near maximal in the absence of added Ca\(^{2+}\) and calmodulin (only contaminating calcium was present, which gave a measured pCa of 7). This indicated that the protein kinase responsible for the effects on NMDARs is activated by very low calcium concentrations.

These results show that each glutamate receptor subtype is selectively associated in the PSD to a different type of protein kinase, of which CaMKinase II is the only one that could be indirectly characterized. The general PKC inhibitor, 1 \(\mu\)M Bisindolylmaleimide I (BisI), was incapable of changing the effects of phosphorylation on the activity of NMDARs (not shown), thus suggesting that PKC is not involved in these effects, at least under the present experimental conditions, i.e. without neuronal cytosolic enzymes present.

### 3.4. Functional association of protein phosphatases to the PSDs

Finally, some evidence for the presence of functional protein phosphatases in our PSD preparation is shown in Fig. 7. AMPAR currents activated by quisqualate could be enhanced by the addition of phosphorylation conditions to the recording bath, as described. The subsequent addition of the general phosphatase inhibitor, NaF (4 mM), produced a two- to three-fold increase in AMPAR currents in an experiment that lasted about 15 min. On the other hand, when the phosphorylation conditions were washed out, AMPAR activation diminished gradually, as shown in Fig. 8.

### 4. Conclusions

Our recordings of iGluRs in isolated patch-clamped PSDs, in which receptor function is preserved, validate this
system for the study of the intricate molecular mechanisms thought to be involved in their physiological regulation. Regarding iGluR subtypes, kainate receptors have been difficult to record because they induce small and rapidly desensitizing currents [30,96]. In this case, we think that we have been able to record functional kainate receptors because our PSD preparation contains the kainate subunits KA2 [137] and GluR6/7 (Wyneken, unpublished results). Also, the kainate concentrations used in these studies (0.2 to 2 μM) are in the selective range for this receptor subtype [61], and finally, kainate receptors were activated by the addition of ATP–Mg²⁺ to the recording solution in the presence of EGTA, a condition that was never able to activate AMPARs.

In relation to voltage-dependent (Mg²⁺)₀ block of NMDA receptors, there is no agreement regarding the effect of phosphorylation on this key control mechanism. Thus, in isolated trigeminal neurons, intracellular perfusion of PKC led to a reduction of the blocking effect of low (30 μM) (Mg²⁺)₀ [16]. In cultured rat cortical neurons, stretch-induced injury was found to markedly reduce the blocking effect of (Mg²⁺)₀, inhibition [148] and in excised patches of CA1 hippocampal neurons, it is suggested that a decreased (Mg²⁺)₀ inhibition may be responsible for NMDAR potentiation by the protease thrombin, that should be mediated by kinases and/or metabotropic receptors [33]. On the other hand, in isolated hippocampal neurons, although PKC potentiates NMDA receptor activity, it does not affect (Mg²⁺)₀ block [141]. This is also seen in cloned NMDA receptors expressed in Xenopus oocytes [132], while in cerebellar granule cells PKC inhibits NMDA receptor function without changing their sensitivity to (Mg²⁺)₀ [112]. We have tried to solve this contradictory findings by further exploring the effect that phosphorylation of isolated, patch-clamped PSDs, by the endogenous protein kinases present in them, has on the blocking action of (Mg²⁺)₀ present at physiological concentration. In this preparation, it has been reported that phosphorylation is indeed capable of increasing NMDA receptor currents at negative membrane potentials even in the presence of (Mg²⁺)₀ [136]. The results obtained at mM, physiological concentrations of (Mg²⁺)₀, indicate that phosphorylation of PSD proteins by endogenous kinases is able to greatly relieve the blocking effect of this cation [92]. Such unblocking shall enhance ion flow, especially Ca²⁺, through the NMDA receptor ion channels, even at quite negative membrane potentials. This has an important physiological significance, because NMDA receptors, unblocked by phosphorylation, become less dependent for activation on the simultaneous depolarization of non-NMDA receptors and, even at resting potential (ca. – 70 mV), their stimulation by glutamate shall lead to a substantial amount of Ca²⁺ influx into dendritic spines. This self-sustaining NMDAR activity may help trigger the many plasticity mechanisms in which this receptor type is involved.

The precise nature of the protein kinases involved, as well as the proteins whose phosphorylation leads to the unblocking, remains, however, undefined and open to further research. The effects of phosphorylation observed here are not due to a decrease in the tonic inhibition by (Zn²⁺)₀ of NMDA receptors [82], because our recording solution contained 0.1 mM EDTA, that chelates any contaminating Zn. This result is similar to that seen with Src potentiation of hippocampal NMDARs [142].

The present study thus supports the initial findings of Chen and Huang [16], obtained in trigeminal neurons, extending them to physiological levels of (Mg²⁺)₀, as well as to the whole brain cortex and hippocampus, from which the PSDs were derived. The differences with other studies, in which NMDAR phosphorylation did not change voltage-dependent block by (Mg²⁺)₀, may possibly be explained because these negative results were obtained using patch-clamped cortical or hippocampal neurons, in which the NMDARs are present mainly in dendritic spines that are distant from the cell body, where the clamp electrode is present. This would not allow adequate voltage control of them. It also seems possible that in those studies, most of the receptor currents recorded are generated at extrasynaptic NMDARs present in the soma and proximal dendrites, that lack the associated proteins that allow, when phosphorylated, the relief of (Mg²⁺)₀ block. On the other hand, isolated nucleus caudalis trigeminal neurons are small, i.e. 10–20 μm in diameter, and their primary dendrites relatively short (Huang, personal communication), thus allowing an adequate voltage control of the synaptic NMDARs presumably present in them. In the present work, by definition, only synaptic receptors, i.e. those present in the PSD, are recorded, and these are located at the tip of the patch pipette, thus allowing an excellent voltage clamp. The differences, regarding the effect of phosphorylation on voltage-dependent Mg²⁺ block, between synaptic (Ref. [16] and present work) and extrasynaptic or cloned NMDARs (Table 1), could also be explained if only synaptic receptors, incorporated into PSDs, were coupled to the specific kinase, we have now described, which, when activated, leads to the unblocking effect.

The fact that the general protein phosphatase inhibitor NaF enhanced greatly the AMPAR currents following PSD phosphorylation confirms that GluR function in PSDs is tightly controlled by the opposing activities of phosphatases and kinases, and also that, in the PSD, protein phosphatases are highly active.

5. Physiological implications

The results shown above, when extrapolated to in vivo conditions, have many physiological implications. Thus, as the concentration of ATP may be presumed to be nearly constant in dendritic spines, where PSDs are mainly present, the enhancement by phosphorylation of iGluR activity shall be a function of the conditions that activate
each of the protein kinases, which we have shown to be selectively associated to the different glutamate receptor subtypes. Thus, kainate receptors, that only need ATP and Mg$^{2+}$ to be activated in the PSD, shall always be maximally activated, and not subject to further activation when (Ca$^{2+}$)$_i$ increases, or to deactivation when it decreases. Thus, synaptic transmission mediated by the postsynaptic kainate receptor subtype is not regulated by changes in (Ca$^{2+}$)$_i$ and its efficacy is always very high. This makes these receptors ideally suited for the unfailing transmission of signals in circuits that are vital for the individual and that may be presumed to be phylogenetically more ancient.

On the other hand, AMPARs, because they are maximally dependent on intraspine calcium concentrations, have a very large dynamic range of activation, which can vary from a low level of activity at pCa 9 to a very high one at pCa 5. This makes this type of glutamate receptor most suited for plasticity. However, we have also shown that protein phosphatases functionally linked to AMPARs are highly active, and that the activation of these receptors is greatly enhanced when the phosphatases are

![Fig. 3](image_url)

Fig. 3. NMDA plus glycine-activated currents show a complex kinetic behaviour and voltage-dependent Mg$^{2+}$ blockade. The ionic composition of the recording solutions was symmetric except that the external recording solution contained 2 mM Mg$^{2+}$. Different time scales of a recording at a membrane potential ($V_m$) of +80 mV (A) and at -80 mV (B). One-minute current amplitude histograms for both membrane potentials are shown in (C). Note the marked current inhibition seen at -80 mV. Mean current values of 1-min recordings are given.
inhibited. This is an indication that the degree of AMPAR phosphorylation is in a steady-state, with phosphoryl groups being added and removed continuously. Also, when (Ca$^{2+}$), decreases, the phosphorylation of the receptor and thus, its state of activity, decreases. We have observed that, when the recording bath after phosphorylation with 2 mM ATP–Mg$^{2+}$, 10 μM calcium and 12.5 μg/ml calmodulin is returned to the control situation, i.e. no ATP–Mg$^{2+}$ and the pCa is 7 (Fig. 8), AMPARs deactivate. This indicates that the phosphorylated state of AMPARs is highly labile, but, this does not deny that persistently activated CaMKII may continue to phosphorylate them, as cogently argued by Lisman et al. [68]. However, as our finding that protein phosphatases are highly active in the PSD does not fit into the current version of the CaMKII memory switch theory [68], we suggest, rather, that additional mechanisms are needed to sustain the increased efficacy during long periods of time. Recently, “upstream” mechanisms have started to be clarified [45,73,74,78,120]. These include increased synthesis and insertion into PSDs of GluRs and other synaptic proteins, which may even increase the size of PSDs, as well as the activation of several plasticity-related genes [89]. It is not clear what triggers these upstream mechanisms, although increased Ca$^{2+}$ in the spines, cyclic AMP, and the activation of different protein kinases and signalling cascades have been shown to be involved [36,39,73]. It is not known whether genomic changes are irreversible, and the genes become permanently activated, although this, by homology to other genes, would seem unlikely, since it would need a mutation-like event. The other non-genomic upstream mechanisms, all of which involve some sort of protein modification, usually a
phosphorylation, are certainly reversible and should revert to the initial state once the stimulus that switched them on, ceases. The half-life for the decay of these genomic and non-genomic mechanisms may be conceived as longer than the one seen in phosphorylated AMPARs, but nevertheless deactivation should occur.

In our opinion, the changes that allow a very long permanence of increased synaptic efficacy cannot exclusively be due to a static structural modification, i.e. the phosphorylation of a protein or the insertion of a greater number of GluRs into PSDs, because all these processes are subject to a turnover, and once the stimulus that led to a modified structural state ceases, turnover shall revert the changed structure to its original state. A stable change in synaptic efficacy would require that some of the initial changes, that occur during synaptic activation, give rise to

Fig. 6. Effects of PSD phosphorylation on iGluR mediated currents. Bars are the mean 1-min currents ± S.E.M. in different experiments (n shown in parenthesis) when compared to controls. The different phosphorylation conditions were: hatched bar: 2 mM ATP–Mg^{2+} in the presence of contaminating Ca^{2+} (pCa 7); white bars: 2 mM ATP–Mg^{2+} plus 0.1 mM EGTA (pCa 9); black bars: 2 mM ATP–Mg^{2+} plus 10 μM Ca^{2+} (pCa 5) plus 12.5 μg/ml calmodulin.

Fig. 7. Inhibition of phosphatases induces further AMPAR activation. Upper panel: the mean current of consecutive 1.024 s of quisqualate-activated receptors in a membrane patch was plotted as a function of time. Three sequential experimental conditions are shown: (I) a control situation in the presence of 1 μM quisqualate in the patch pipette; (II) after addition to the recording bath of 2 mM ATP–Mg^{2+} plus 10 μM Ca^{2+} (pCa 5) plus 12.5 μg/ml of calmodulin; (III) after the addition to the recording bath of 4 mM NaF. Lower panel: Representative recordings for each situation are shown.
a self-sustained dynamic state, in which the activated state is maintained by the continuous re-generation of the conditions present at the beginning of activation. One of these mechanisms has been recently discussed by Si et al. [109,110] who found prion-like properties in the synaptic protein cytoplasmic polyadenylation element binding protein (CPEB). Synaptic activation induces a conformational change that is self-perpetuating.

Our model predicts that the effect of phosphorylation on the activity of NMDARs and, especially, the dependency on low Ca\(^{2+}\) concentrations of the protein kinase selectively associated to them, is another example of a self-sustained mechanism that may keep a glutamatergic synapse in a state of enhanced efficacy for a very long period (Fig. 9).

Thus, once a glutamatergic synapse is potentiated because of an intense release of glutamate, phosphorylation of NMDARs allows them to unblock from voltage-dependent Mg\(^{2+}\) inhibition at substantially smaller depolarizations (cf. Fig. 5) relative to non-potentiated NMDARs. This shall lead, during normal, low frequency operation of the synapse, to an easier opening of NMDAR channels, and to greater Ca\(^{2+}\) influx, able to repotentiate AMPARs. When excitation ceases, intraspine Ca\(^{2+}\) starts to decrease, and so AMPARs progressively deactivate. This does not exclude the operation of mechanisms like those proposed by Lisman [68], where AMPAR deactivation can be counteracted by rephosphorylation by active CaMKII. However, as the NMDAR-associated kinase is active at low (Ca\(^{2+}\))i (i.e. pCa 7), it shall be much less affected by the decreasing Ca\(^{2+}\) concentration, keeping the receptors activated also for long periods. Slow frequency synaptic activation, and even quantal spontaneous glutamate release, could keep feeding

![Fig. 8. Effect of PSD phosphorylation on AMPAR currents. All the recordings were obtained from the same membrane patch. At the left hand side, current records; at the right, 1-min current amplitude histograms are shown. (A) Control recording of a membrane patch at +60 mV in the absence of agonists in the patch pipette. (B) Quisqualate (0.5 μM) was injected into the patch pipette, (C) 2 mM ATP –Mg\(^{2+}\) plus 1 mM EGTA were added to the bath (“intracellular” side of the PSD). (D) 2 mM ATP –Mg\(^{2+}\) plus 10 μM Ca\(^{2+}\) (pCa 5) plus 12.5 μg/ml of calmodulin were added to the bath. (E) 10 min after, the bath fluid was replaced by one without ATP –Mg\(^{2+}\), Ca\(^{2+}\) or calmodulin, that is, similar to the initial one. The control recording solution was 140 mM NaCl, 0.1 μM EDTA, 10 mM HepesNa, pH 7.4. Traces A, B and D are reproduced from [136].](image-url)
Ca$^{2+}$ into the spines in sufficient amounts to keep its concentration at a level that stimulates the “NMDAR kinase”, and thus, keeps the synapse potentiated. Release of Ca$^{2+}$ from intraspine stores could also contribute to this [100]. This is supported by the finding that APV is able to reduce an established hippocampal LTP [140], and, especially by the findings that the resting Ca$^{2+}$ concentration in resting spines can reach 50 to 79 nM (pCas of 7.3 to 7.1) [35,88], which would keep the NMDARs in a state of high responsiveness. The recent findings that subthreshold activation of NMDARs is able to elicit in them significant Ca$^{2+}$ elevations [56], also support our hypothesis. This long-term activation of NMDARs, with its consequent maintenance of an intraspine pCa of 7.1, could also be part of the so-called “tag”, that signals to the genomic products where an activated synapse is, and induces their localization in them [80].

This postulated self-sustaining NMDAR mechanism may be only one of a series of other self-sustaining processes that can contribute to the establishment of an increased efficacy of central glutamatergic excitatory synapses. Such mechanisms seem closely related to Descartes view on brain function, when in his Traité de l’Homme he stated: “When the action induced by the object increases the opening of the tubes (or pores), and causes the (animal) spirits to penetrate in larger amounts than if such increase in opening had never occurred, they will have the strength to form certain passages that shall remain open even after the action of the object has ceased. In case they close, at least the small pores retain a certain disposition to be opened again” [23] (In Descartes, the concept of “animal spirits” was equivalent to our nerve impulses, and we may equate his “small pores” to synapses or, even, to glutamate receptor ion channels.)

In the recent past, our laboratory has shown that synaptic vesicles derived from the rat CNS have a very high glutamate content, and that this amino acid is the only physiologically relevant transmitter that is able to interact with kainate, AMPA or NMDARs, thus establishing the concept of the glutamatergic central synapse (reviewed by Orrego and Villanueva [94]). With this new physiological preparation, the isolated patch-clamped PSD, we now offer a tool of great promise for future research.

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