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Metabotropic and NMDA glutamate receptor interactions with osmotic stimuli in supraoptic neurons

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Abstract

During increases in plasma osmolality, the actions of extrinsic glutamatergic synaptic inputs on magnocellular neuroendocrine cells (MNCs) are thought to combine with intrinsic osmosensitivity of these cells to promote the release of vasopressin (VP). In the present study, changes in intracellular calcium were used as an endpoint to examine putative interactions between osmotic stimuli and NMDA and metabotropic glutamate receptors (mGluRs). Exposure of MNCs to hyperosmotic solutions resulted in a very small, gradual increase in intracellular calcium. NMDA (100–300 μM) combined with osmotic stimulation gave rise to a synergistic increase in intracellular calcium. The broad spectrum mGluR agonist, (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) and the type I mGluR agonist, (*RS*)-3,5-dihydroxyphenylglycine (DHPG) evoked an acute calcium rise followed by a sustained increase. However, when combined with the hyperosmotic stimulus, the calcium responses to 1*S*,3*R*-ACPD and DHPG were suppressed. The type II agonist, (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), and the type III agonist, (+)-2-amino-4-phosphonobutyric acid (AP-4), facilitated the loss of calcium from the MNCs and were largely unaffected by the osmotic stimulus. These osmotic interactions with NMDA and mGluR function not only help to explain the mechanisms that underlie osmotically mediated changes in MNC function, but also have implications for the impact of hyperosmotic stress in various pathological conditions. © 2002 Published by Elsevier Science Inc.

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1. Introduction

Plasma osmolality is normally maintained within tight limits by the actions of circulating vasopressin (VP), which regulates water retention by the kidney (antidiuesis). The release of VP in response to changes in plasma osmolality is dictated by the activity of magnocellular neuroendocrine cells (MNCs) in the supraoptic and paraventricular hypothalamic nuclei. Two osmosensitive processes are thought to converge at these cells to control the level and nature of the cellular activity. Osmosensitive cells are thought to reside outside the blood–brain barrier within the organum vasculosum of the lamina terminalis (OVLT) (Bourque et al., 1994; Johnson, 1985). Increases in plasma osmolality activate these cells, which, in turn, synaptically activate the MNCs directly and indirectly (Camacho and Phillips, 1981; Sladek and Johnson, 1983), in part, via glutamatergic synapses (Richard and Bourque, 1992). In addition, the MNCs are intrinsically osmosensitive. Stretch-inactivated mechanoreceptors in the membranes of MNCs respond to increases in plasma osmolality by opening a nonselective cation channel (Oliet and Bourque, 1993). Opening of the channel results in a small depolarization of the cell, which alone is not sufficient to generate a action potentials (Oliet and Bourque, 1993). Instead, the mechanosensitivity is thought to facilitate activation of the MNCs by extrinsic synaptic inputs (Bourque et al., 1994).

The most prominent excitatory synapses that regulate activity within the MNCs are glutamatergic (Meeker et al., 1993; Van den Pol, 1991). These glutamatergic synapses terminate on the cell body and dendrites of MNCs, which express each major subtype of glutamate receptors, including NMDA, AMPA and metabotropic glutamate receptors (mGluRs) (Meeker et al., 1994, 1999). All of these receptor subtypes appear to play a role in the activation of the MNCs. Activation of the NMDA receptor in particular is thought to be responsible for the generation of bursting patterns, which develop on calcium-dependent plateau potentials (Andrew

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et al., 1989; Theodosis et al., 1983) and are associated with hormone release (Hu and Bourque, 1992; Nissen et al., 1994).

In addition to NMDA, release of VP can be stimulated by the mGluR agonist, ACPD (Meeker et al., 1999; Morsette et al., 2001). The mRNA for several mGluRs are expressed in the MNCs in the following relative amounts: mGluR3 > mGluR1>mGluR7 (Al Ghoul et al., 1998). These receptors have complex functions acting both presynaptically at glutamatergic and GABAergic synapses (Schrader and Tasker, 1997a), as well as through activation of inward currents at the MNC soma (Schrader and Tasker, 1997b). The excitatory actions of *trans*-ACPD, which stimulate VP release, are thought to be due to actions at type I mGluRs (Morsette et al., 2001). In vitro, the broad spectrum agonists *trans*-ACPD or (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD) stimulate increases in intracellular calcium in the MNCs (Hattori et al., 1998; Meeker et al., 1999). In addition to activation of VP release, NMDA, 1*S*,3*R*-ACPD and osmotic stimuli all rapidly and independently increase the expression of a c-Jun-N-terminal protein kinase (Meeker and Fernandes, 2000). Thus, intrinsic osmotic effects and extrinsic glutamatergic synaptic stimulation of NMDA and metabotropic glutamate receptors, each capable of activating the MNCs, converge during increases in plasma osmolality. How these stimuli might interact is unknown but is a question important for the understanding of the physiology of these and other cells exposed to hyperosmotic stress. In the present series of studies, we sought to determine if and how intrinsic osmotic stimulation modulates the responses to NMDA and mGluR stimulation. The fluorescent dye, Fluo-3, was used to measure changes in intracellular calcium during exposure to an osmotic stimulus in combination with NMDA or an agonist for each major mGluR subtype. Osmotic stimulation was found to have potent but opposite influences on intracellular calcium increases induced by NMDA or metabotropic glutamate receptors.

2. Materials and methods

2.1. Tissue culture

The region of the supraoptic nucleus (SON) was cultured from E17 to E18 fetal rat brain in the form of a punch culture (Meeker et al., 1999). Fetuses were removed from pregnant female Long–Evans rats immediately postmortem and transferred to sterile HEPES-buffered Hank's balanced salt solution (HBSS), pH = 7.4. Procedures for harvesting the fetuses have been reviewed and accepted by an Institutional Animal Care and Use Committee, and are in compliance with NIH Guidelines for the Care and Use of Laboratory Animals. The brains were removed, washed extensively in sterile HBSS and then transferred to sterile complete medium (minimum essential medium (MEM) + 10% fetal bovine serum + 20 μg/ml gentamicin). A specially prepared 25-gauge needle was then used to "punch" the region of the SON from the brain. The core of tissue was placed on a 31-mm round coverslip previously coated with poly-D-lysine and covered with a thin film of medium. After approximately 2–4 h, the tissue attached to the coverslip and a supplemental feeding of medium was given. These tissue punches maintained some of the integrity of the SON and eliminated much of the trauma associated with enzyme treatment and trituration in dissociated cultures. The neurons in the punch migrated into the surrounding region to provide a discrete field of neurons easily identified for imaging studies. Morphological features typical of magnocellular neurons developed over a period of approximately 6–10 days in vitro. Cells were fed with complete medium every 2 days.

2.2. Digital calcium imaging and morphometry of MNCs

Cultured cells were washed free of serum and are loaded with the calcium sensitive dye, Fluo-3 AM, by incubating in a 2 μM solution in the tissue culture incubator for 40 min at 36°. MEM was used for the loading medium. After loading, the cultures were washed twice in aCSF and the coverslips transferred to the flow cell for imaging. Artificial CSF (aCSF) composed of the following salts (in mM): NaCl (137), KCl (5.0), $CaCl_2$ (2.3), $MgCl_2$ (1.3), HEPES (10), glucose (20); pH = 7.3–7.4, 290 mosM/kg, was perfused across the coverslip at a rate of approximately 1 ml/min (except for chronic stimulation experiments). All experiments were run at room temperature (25 °C). MNCs were identified based on size and morphology at a final magnification of $\sim 1200\times$ and images of the cell and basal fluorescence were collected using a high resolution Princeton Digital CCD Camera coupled to a Metamorph Imaging System (Universal Imaging). Drug was applied to the cells from a pipette positioned above the cell using a Picospritzer at a pressure of 2.5 psi with a duration of 2 s. Perfusion of the cells with aCSF immediately followed drug application to insure rapid washout of the drug. For the osmotic stimulation experiments, the osmotic stimulus was typically applied for the duration of the experiment. Digital images were saved every 6–10 s for a period of 6–10 min. Analysis was accomplished by recovering each image from memory and measuring the brightness level (fluorescence) within each cell at each time point. The increase in fluorescence intensity within each cell was corrected for background and evaluated relative to the prestimulation baseline at $t = 0$ to correct for cell to cell differences in dye loading and intrinsic fluorescence ($\Delta F = F_{\text{stimulated}}/F_{\text{baseline}}$). The relative changes from baseline were then expressed as percent increases or decreases $[(\Delta F \times 100) - 100]$. Cells expressing very weak fluorescence or very high fluorescence indicative of poor loading or damage were excluded from the experiments.

Curves of $\%\Delta F$ vs. time were evaluated for both acute and delayed responses, as well as rate of recovery. To

provide a conservative analysis of the changes in intracellular calcium, peak increases from baseline were determined for each cell and used for statistical analysis. This measure generally matched the temporal pattern illustrated in the figures and was supplemented with additional analyses of secondary peaks and recovery rates where appropriate. Recovery rates were calculated from the slope of the plot of ln $\%\Delta F/\%\Delta F_{peak}$. Peak Ca^{2+}, accumulation and recovery rates for each cell were pooled within each group and analyzed by *t*-test. Rate processes (e.g., regression analysis) were analyzed with Graphpad curve fitting software.

2.3. Drug and osmotic stimulation

N-Methyl-D-aspartic acid (NMDA) was obtained from Sigma (St. Louis, MO) and the mGluR agonists, 1*S*,3*R*-ACPD, (*RS*)-3,5-dihydroxyphenylglycine (DHPG), (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV) and L(+)-2-amino-4-phosphonobutyric acid (AP-4) were obtained from Tocris-Cookson (Ellisville, MO). Water soluble drugs were dissolved in aCSF. Drugs with poor aqueous solubility were first dissolved in dimethyl sulfoxide (DMSO) at a 1000-fold stock concentration followed by dilution in aCSF to give the working concentration. Osmolality of the aCSF was 290 ± 2 mosM/kg to match the osmolality of rat plasma based on measurements of osmolality with a Wescor (Logan, UT) vapor pressure osmometer. Hyperosmotic aCSF was made by adding tissue culture grade NaCl to the aCSF. Chronic stimulation of the cells with drugs was achieved by transferring the MNCs to the microscope in the imaging chamber containing normal aCSF. Three images were collected for baseline fluorescence determination. For chronic stimulation, the fluid was then aspirated quickly and replaced immediately by aCSF containing drug, 20 mosM/kg excess NaCl or drug + 20 mosM/kg excess NaCl. Mg^{2+}-free aCSF was made by eliminating the $MgCl_2$ from the aCSF and adding back an equivalent number of osmoles of NaCl. Acute stimulation of the MNCs with drug was accomplished by placing the cells in normal or hyperosmotic aCSF in the imaging chamber and then transferring the cells to the microscope. A constant flow of normal aCSF or hyperosmotic aCSF was then started at a rate of approximately 1 ml/min and three baseline images were collected. The flow was briefly stopped, drug applied using a glass pipette and a 2-s pressure pulse from a Picospritzer. Flow was then re-initiated for the duration of the experiment. Images were collected every 10 s following drug stimulation for a period of 10 min.

3. Results

When cultured MNCs are challenged chronically with an aCSF solution containing 20–30 mosM/kg excess NaCl, they showed a very small and gradual increase in intracellular calcium (Fluo-3 fluorescence) beginning approximately 3 min after the initial exposure (Fig. 1A). After approximately 6 min, the increase in fluorescence stabilized at approximately 10% above baseline. In a similar fashion, cultures that were pretreated with hypertonic aCSF had basal fluorescence levels (445.1 ± 24.1 brightness units) that were 17.8% higher than corresponding controls (378.0 ± 20.4 brightness units). Chronic application of 300 μM NMDA to similar cultures resulted in an immediate 14% increase in intracellular calcium followed by a slow rise for the next 2 min, which stabilized at approximately 25% above baseline. The same stimulus (300 μM NMDA) applied to the cultures in a hypertonic environment (+20 mosM/kg) resulted in an immediate average increase of 28% followed by a gradual rise to 70% above baseline. The peak increase was almost two-fold greater than the predicted additive effect of NMDA and hypertonic stimulation suggesting a strong synergism between the two stimuli ($t = 2.14$, $df = 33$, $P = .0402$, NMDA + osm vs. NMDA). Since the osmotic effect did not reverse within the duration of this experiment, a second experiment was conducted to examine the persistence of the effect over a period of 60 min. As illustrated in Fig. 1B, intracellular calcium continued to rise above control values for the duration of the experiment ($t = 3.25$, $df = 49$, $P = .0021$). Combined stimulation with hyperosmotic (+20 mosM) aCSF and NMDA at 100 μM resulted in a continuous increase that was significantly greater than the osmotic stimulation alone ($t = 2.29$, $df = 43$, $P = .0269$). A synergistic osmotic effect on intrinsic NMDA receptor activity was also seen when the MNCs were challenged with an osmotic stimulus in Mg^{2+}-free aCSF (Fig. 1C). An initial average increase of approximately 10% was followed by a gradual accumulation of calcium to approximately 27% above baseline ($t = 2.68$, $df = 63$, $P = .0094$) after 10 min. Magnesium-free medium alone resulted in a gradual decline in intracellular calcium followed by a small, gradual increase after approximately 4 min. To more closely evaluate the effects of the hyperosmotic stimulus on NMDA receptor stimulation, intracellular calcium changes were measured following a brief pulse of 100 μM NMDA (Fig. 1D). Control cultures stimulated with 100 μM NMDA showed a small, immediate elevation of intracellular calcium that returned to baseline with a slight plateau apparent in the recovery at 130–160 s poststimulation. The same stimulus in hyperosmotic aCSF resulted in a larger acute response to NMDA from 17% in the control to 27%, which just failed to reach significance ($t = 1.76$, $df = 72$, $P = .0821$). However, the osmotic stimulus also provoked a secondary rise in intracellular calcium with a peak approximately 80–100 s after the initial rise. This secondary peak was significantly greater than temporally matched calcium increases in response to NMDA ($t = 3.66$, $df = 72$, $P = .0005$). Following the second rise, intracellular calcium rapidly returned to baseline values with the same kinetics as the initial recovery rate from the peak control (NMDA alone) response. Examples of the acute

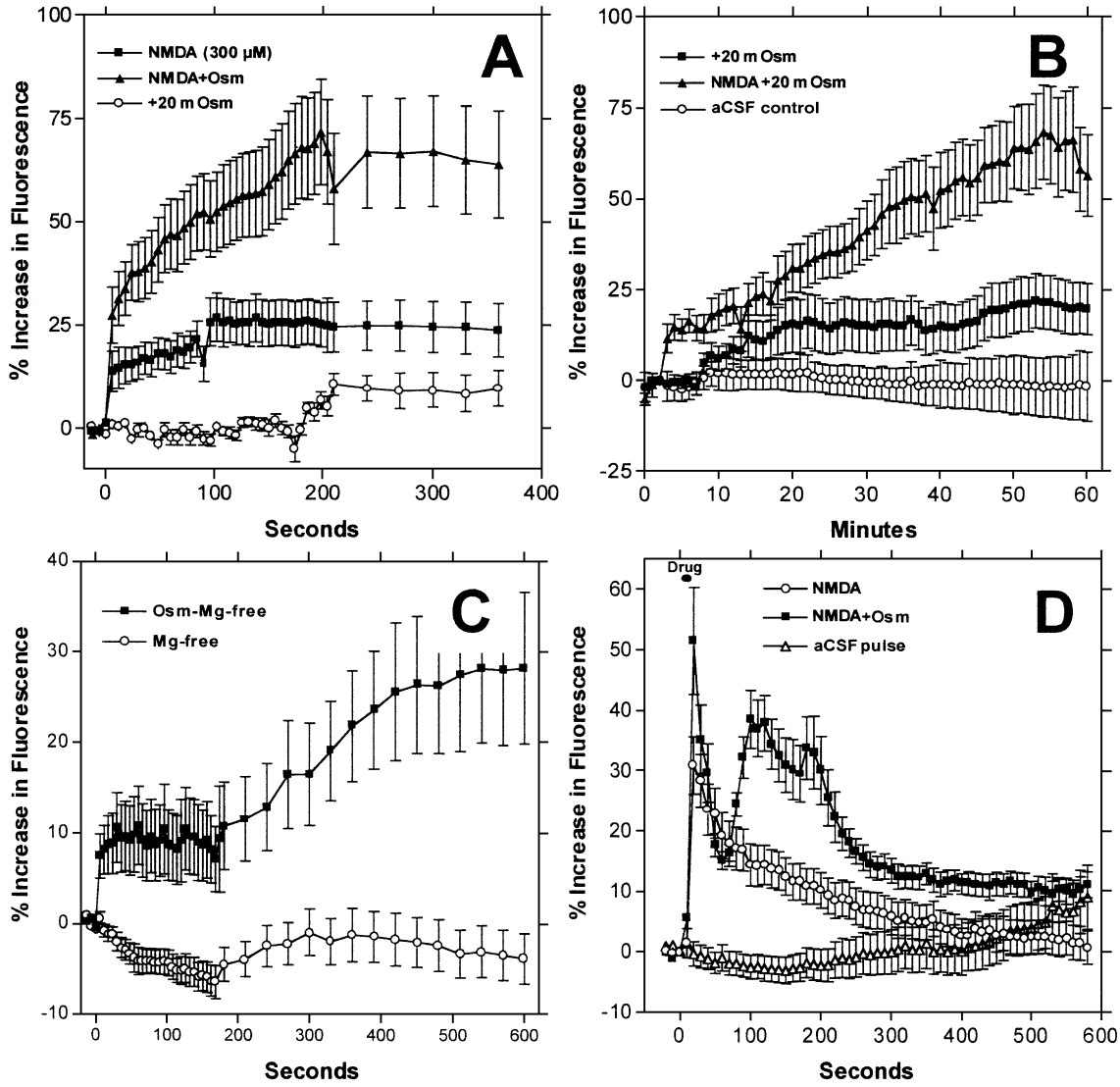

Fig. 1. Interaction between NMDA and a hyperosmotic stimulus. (A) Cultured MNCs were incubated in aCSF containing 300 μM NMDA ($n=23$), 20 mosM/kg excess NaCl (+20 mosM, $n=9$) or NMDA + osmotic stimulation ($n=20$). Each stimulus was applied at $t=0$ s after collection of three prestimulation baseline images and remained for the duration of the experiment. Osmotic stimulation alone resulted in a small delayed increase in intracellular calcium as measured by the percent increase in fluorescence. NMDA resulted in an immediate increase in intracellular calcium that rose to a peak of approximately 27% and remained elevated throughout the duration of the stimulus. The combination of NMDA + osmotic stimulation resulted in an increased immediate response followed by a steeper rise to a peak approximately 70% above baseline. (B) The increases in intracellular calcium in response to the chronic application of the osmotic stimulus or NMDA + osmotic persisted for at least 1 h. Vehicle controls showed negligible changes in intracellular calcium. (C) MNCs placed in Mg^{2+}-free aCSF containing 20 mosM/kg excess NaCl (osM-Mg-free, $n=16$) showed an immediate increase in intracellular calcium followed, after 240 s, by a continuous rise to approximately 28% above basal. Incubation of cells in isoosmotic, Mg^{2+}-free aCSF at 290 mosM/kg ($n=13$) resulted in a slow decline, which began to rise slightly after 240 s. (D) A brief (2 s) pulse of NMDA (100 μM) applied to cultures exposed to hypertonic medium ($n=17$) resulted in a rapid rise followed by a significant second rise relative to NMDA alone ($n=60$). Cells were transferred to normal or hyperosmotic aCSF approximately 10 min prior to data collection. A similar pulse of aCSF ($n=15$) produced negligible changes in intracellular calcium. All data points represent the mean ± S.E.M. across all cells at each time point.

and delayed intracellular calcium changes in individual MNCs exposed to NMDA or NMDA + osmotic stimulation are illustrated in Fig. 2.

To evaluate if a similar osmotic effect could be seen with mGluR stimulation, a brief pulse of 1*S*,3*R*-ACPD was applied to the cells in hypertonic or normal aCSF. ACPD (100 μM) evoked a small rapid rise in intracellular calcium followed by recovery to values approximately 6% above baseline (Fig. 3A). The same stimulus in the presence of hypertonic aCSF produced a similar rise. Recovery to baseline was not significantly different for the two curves. To evaluate the mGluR subtypes responsible for the increase in intracellular calcium as well as the sensitivity of each subtype to osmotic stimulation, agonists with specificity for types I, II and III mGluRs were applied to the MNCs in normal or hypertonic aCSF. At 30 μM, the type I agonist,

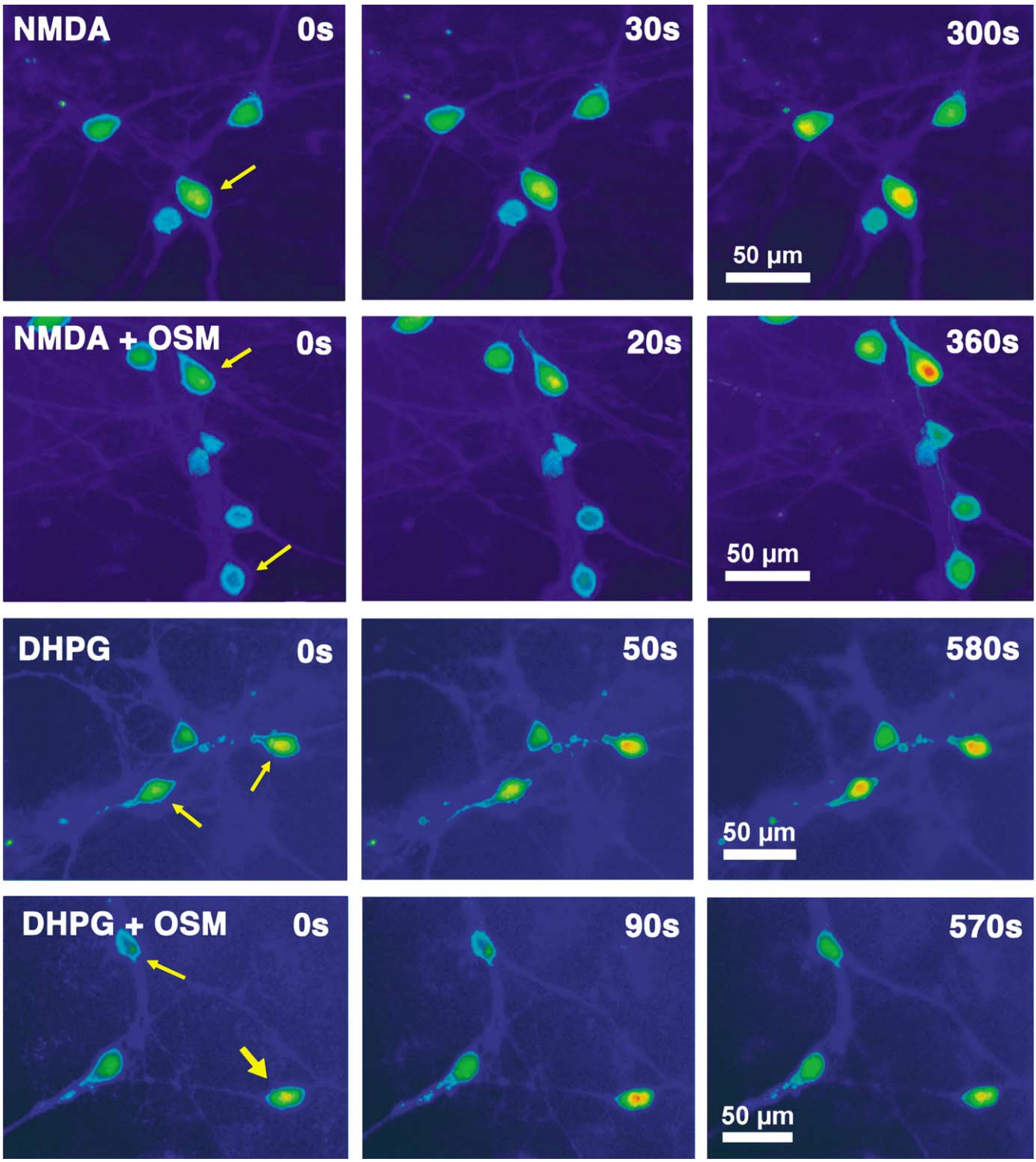

Fig. 2. Example of changes in intracellular calcium in individual MNCs prior to and during stimulation with NMDA, NMDA in hyperosmotic aCSF (NMDA + osm), DHPG or DHPG in hyperosmotic aCSF (DHPG + osm). MNCs were characterized by large oval or rounded cell bodies (arrows) with two or three prominent dendrites. In the presence of NMDA, four MNCs can be seen that show small acute increases in fluorescence at 30 s, followed by a gradual build up of calcium over the next 300 s. Variability in the intensity of the response can be seen between individual neurons. NMDA in the presence of a hyperosmotic stimulus (NMDA + osm) resulted in a larger, more visible acute increase in intracellular calcium in most neurons followed by a larger late rise. Acute (2 s) stimulation of MNCs (arrows) with DHPG resulted in a small acute rise that was maintained for the duration of the experiment. In the presence of a hyperosmotic stimulus, addition of DHPG (DHPG + osm) resulted in an acute increase in some cells, which was maintained for up to 570 s (small arrow) or, less typically, was followed by a return to control levels (large arrow).

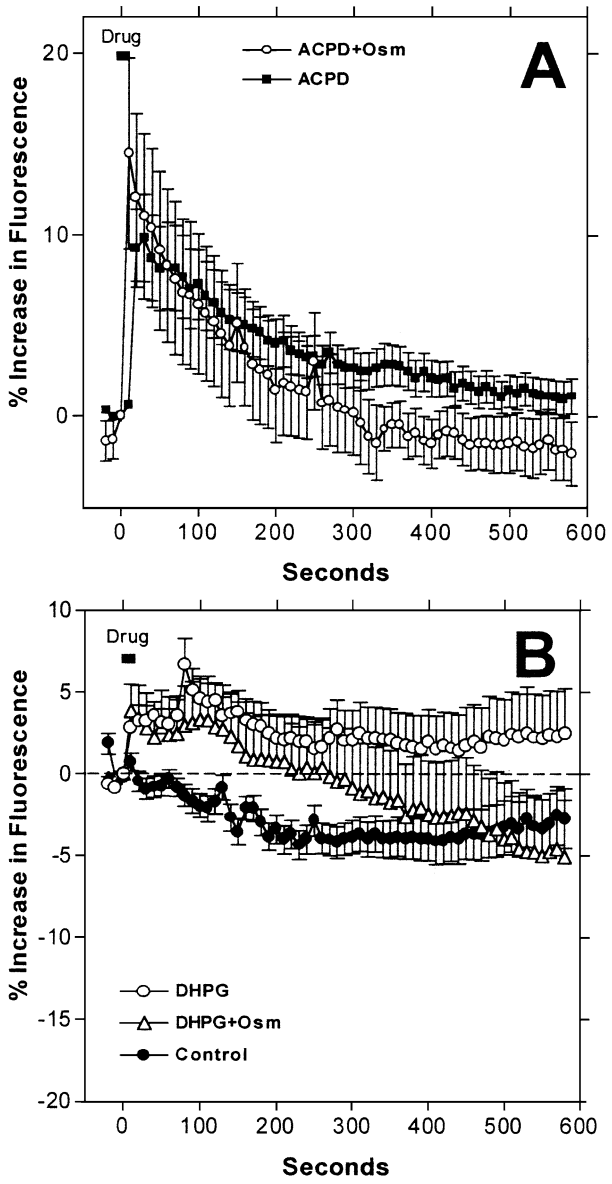

Fig. 3. Intracellular calcium responses of MNCs following an acute (2 s) stimulation of type I mGluRs. (A) Cells were transferred to normal or hyperosmotic aCSF approximately 10 min prior to data collection and then stimulated with 100 μM 1*S*,3*R*-ACPD ($n=20$) or 1*S*,3*R*-ACPD in the presence of hyperosmotic aCSF ($n=48$). A rapid, transient 10% increase in intracellular calcium (percent increase in fluorescence) was seen with 1*S*,3*R*-ACPD followed by recovery. Stimulation with 1*S*,3*R*-ACPD in hyperosmotic aCSF (1*S*,3*R*-ACPD + osm) resulted in a similar acute peak (+14%), which recovered to baseline levels with the same kinetics as 1*S*,3*R*-ACPD alone. (B) Acute (2 s) stimulation with 30 μM DHPG ($n=60$) or DHPG in the presence of hyperosmotic aCSF ($n=39$) both resulted in a small 5–7% increase in intracellular calcium. Controls ($n=61$) run in the absence of drug and in parallel with the series of specific mGluR agonists are shown for reference and showed a slight decrease in fluorescence that stabilized 3–4% below the initial baseline. Values represent the mean ± S.E.M.

DHPG, induced a small average rise in intracellular calcium that peaked 80 s after stimulation (Fig. 3B), dropped slightly and then remained 2–3% above baseline. The increase, although small, was significantly greater ($t=3.10$, $df=121$, $P=.0024$) than the changes in intracellular calcium in vehicle-stimulated control cultures that were run in parallel with the series of mGluR agonists. Individual neuronal responses tended to be considerably larger than the mean since many cells failed to respond to the DHPG. Stimulation with DHPG in hyperosmotic aCSF resulted in a similar slow average acute rise in intracellular calcium to about the same

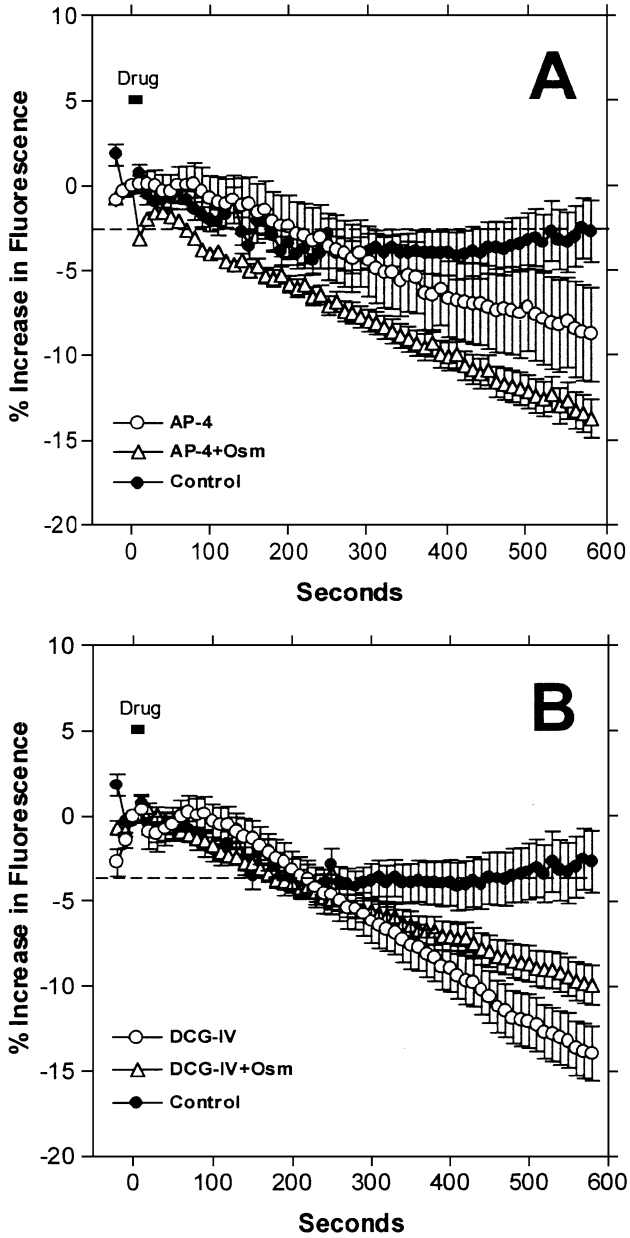

Fig. 4. Intracellular calcium responses of MNCs following an acute (2 s) stimulation with type II or type III agonists. (A) Application of 30 μM DCG-IV ($n=36$) or DCG-IV in the presence of hyperosmotic aCSF ($n=44$) failed to produce significant increases in intracellular calcium. However, after approximately 90 s, a rapid, linear decline in intracellular calcium was observed for the duration of the experiment that was not apparent in the controls. (B) Acute (2 s) stimulation with 10 μM AP-4 ($n=14$) or AP-4 in the presence of hyperosmotic aCSF ($n=39$) also failed to increase intracellular calcium and resulted in a rapid decline in calcium after approximately 90 s. Values represent the mean ± S.E.M.

level. The apparent increase in the rate of recovery in the hyperosmotic solution was largely due to a slight greater number of nonresponding cells. Neurons responding to ACPD in the presence of the osmotic stimulus generally retained the elevated calcium throughout the experiment. Since the response to DHPG was weak, several studies were done at higher concentrations (150 μM). However, responses to the increased concentration were not significantly greater and never reached the level seen with ACPD. Examples of individual MNC calcium responses to DHPG are illustrated at the bottom of Fig. 2. The type II agonist, DCG-IV (30 μM), and the type III agonist, AP-4 (10 μM), both failed to evoke a rise in intracellular calcium (Fig. 4A and B). Instead, a rapid linear decline in intracellular calcium was seen starting 90 s after stimulation. The rate of decline in the presence of either DCG-IV or AP-4 was approximately equal to (AP-4 mean slope = -0.0157 ± 0.0029 s^{-1}, $t = 0.227$, $df = 56$, $P = .8210$) or slightly greater (DCG-IV mean slope = -0.0291 s^{-1} ± 0.0019, $t = 1.12$, $df = 85$, $P = .2653$) than the initial control rate of decline (-0.0135 ± 0.0056 s^{-1}). However, after approximately 3 min, the rate of decline for both DCG-IV ($t = 7.03$, $df = 77$, $P < .0001$) and AP-4 ($t = 3.19$, $df = 56$, $P = .0023$) continued at a significantly greater rate than the control rate that had stabilized at an average positive slope of $+0.1403 \pm 0.0054$ s^{-1}. The rate of decline in the drug-treated cells approximated estimates of the rate of decline of Fluo-3 fluorescence due to quench (0.0362 s^{-1}) indicating the loss of an influence that tends to elevate steady-state levels of intracellular calcium. Hyperosmotic medium decreased the rate of DCG-IV-induced calcium decline slightly from an average of -0.0198 to -0.0291 min^{-1} ($t = 4.209$, $df = 78$, $P < .0001$). The rate of decline in intracellular calcium induced by AP-4 was not significantly affected by the addition of a hypertonic stimulus ($t = 1.34$, $df = 53$, $P = .1872$), although the decrease began approximately 40 s sooner with the combined stimulus. Together, these results suggest that activation of types II and III mGluRs may facilitate the loss of intracellular calcium. To determine if the principal effect of intrinsic mGluR stimulation was to facilitate or suppress calcium mobilization via glutamate receptor activation, the mGluRs were blocked by the broad spectrum antagonist, (+)MCPG, and the cells were stimulated with 100 μM glutamate. As illustrated in Fig. 5, the presence of (+)MCPG greatly facilitated the calcium increase induced by glutamate ($t = 2.48$, $df = 105$, $P = .0145$). Stimulation with (+)MCPG alone failed to have a significant effect on intracellular calcium.

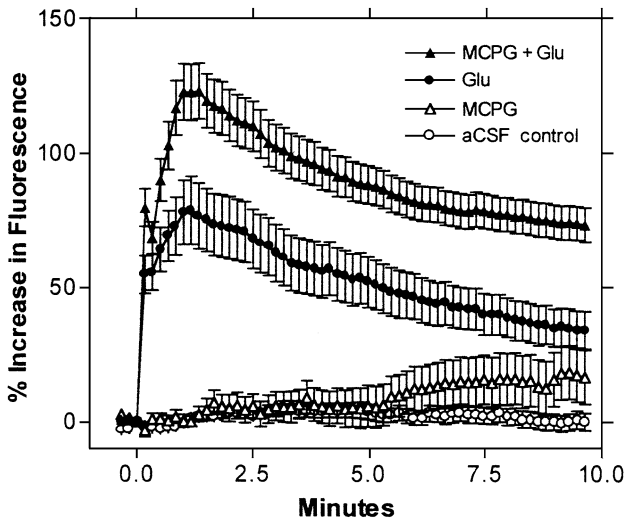

Fig. 5. Incubation of the MNCs with the broad spectrum mGluR antagonist, (+)MCPG (200 μM) resulted in a significant elevation of glutamate-induced increases in intracellular calcium (MCPG + Glu, $n = 46$) relative to stimulation with 100 μM glutamate alone (Glu, $n = 25$). Stimulation with (+)MCPG alone (MCPG, $n = 20$) did not significantly influence intracellular calcium relative to aCSF controls ($n = 25$). Glutamate and (+)MCPG were chronically applied to the cultures. Values represent the mean ± S.E.M.

4. Discussion

Studies by Oliet and Bourque (1993) have shown that MNCs are osmosensitive due to the presence of stretch-inactivated mechanoreceptor channels on the cell surface. During shrinkage, in response to hyperosmotic stimuli, the mechanoreceptor channels nonselectively gate cations. While the cation flux does not result in a substantial depolarization of the cell, it was hypothesized that this stimulus could facilitate excitatory stimulation. Such a facilitation would be particularly important for NMDA receptor stimulation, which is voltage-sensitive and is thought to underlie the development of bursting patterns (Hu and Bourque, 1992; Nissen et al., 1994) associated with hormone release (Bicknell and Leng, 1981; Haller and Wakerley, 1980). In addition, the recently described participation of metabotropic receptors in the activation of VP cells (Schrader and Tasker, 1997b) and release of hormone(Morsette et al., 2001) suggested that osmotic stimulation might also influence the function of mGluRs. To test these possibilities, we monitored changes in intracellular calcium levels in MNCs in vitro after exposure to NMDA or mGluR agonists in the presence or absence of a hyperosmotic stimulus. Hyperosmotic stimulation was found to greatly facilitate the activation of NMDA receptors, while at the same time having little effect on calcium mobilization by mGluRs.

4.1. Osmotic–NMDA receptor interactions

Osmotic stimulation alone resulted in only a very small but significant accumulation of intracellular calcium. However, when combined with NMDA, a substantial enhancement of the calcium response was seen relative to NMDA alone. The synergistic relationship between NMDA and osmotic stimulation during chronic and acute stimulation supports the idea that mechanoreceptor activation may "prime" these cells for stimulation by excitatory synaptic inputs. The osmotic enhancement of intracellular calcium in Mg^{2+}-free medium also indicates that an osmotic stimulus

can facilitate endogenous synaptic activation of the MNCs. This relationship between osmotic and NMDA receptor activation is consistent with the ability of NMDA receptor antagonists to block osmotically stimulated release of VP (Swenson et al., 1998). Various mechanisms could potentially contribute to this synergistic effect. One possibility is a direct osmotic facilitation of NMDA receptor function. The NMDA receptor has been reported to be osmosensitive, but studies by Paoletti and Ascher (1994) indicated that hyperosmotic stress inhibited activation of the receptor. In addition, our results show that the hyperosmotic stimulus provoked the development of a second peak of intracellular calcium that contributed to the maintenance of higher calcium levels within the cells. This second peak appeared 100 s after stimulation and did not begin to recover until 190 s after stimulation. The rapid and complete recovery after the second peak with kinetics that matched the initial recovery from NMDA alone indicated that the calcium recovery process was largely unaltered. The source of this second peak is unknown, but it suggests the contribution from additional mechanisms. One possibility was the osmotic activation of metabotropic glutamate receptors since these receptors have been shown to synergize with NMDA receptors in a variety of neuronal systems under normal (Bashir et al., 1993; Breakwell et al., 1996; Fitzjohn et al., 1996; Musgrave et al., 1993; Pisani et al., 1997; Yang et al., 1998; Zheng and Gallagher, 1992) and pathological (McDonald and Schoepp, 1992) conditions. In addition, mGluR agonists often display a biphasic increase in intracellular calcium with a time course similar to the second peak (Bianchi et al., 1999; Partridge and Valenzuela, 1999). However, as discussed below, osmotic stimulation failed to facilitate calcium mobilization by mGluR agonists, making it unlikely that the mGluRs were responsible for the second peak. Other downstream actions of the osmotic stimulus such as activation of protein kinases could play a role (Meeker and Fernandes, 2000) in the delayed response.

4.2. Osmotic–MGluR interactions

The response to 1*S*,3*R*-ACPD reinforced the view that mGluRs participate in the mobilization of intracellular calcium in MNCs (Hattori et al., 1998; Meeker et al., 1999). The response was characterized by an acute increase followed by a sustained elevation in intracellular calcium. Similar activation of MNCs with *trans*-ACPD in other studies was shown to result in an increase in the excitability of the MNCs due to the inhibition of a resting, voltage-gated and calcium-dependent potassium currents (Schrader and Tasker, 1997b). Similar calcium responses to 1*S*,3*R*-ACPD have also been seen in other neuronal systems (Bianchi et al., 1999; Partridge and Valenzuela, 1999). In these studies, the initial release was attributed to nonvoltage dependent release from IP_3-sensitive stores while the sustained component was attributed to subsequent calcium-sensitive release (calcium-induced calcium release) (Partridge and Valenzuela, 1999), the opening of voltage-gated calcium channels (Bianchi et al., 1999) or activation of a calcium-activated nonselective inward cationic current (ICAN) (Congar et al., 1997). In MNCs, calcium-activated inward currents have been seen following stimulation with *trans*-ACPD (Schrader and Tasker, 1997b). In addition, opening of high voltage-activated calcium channels followed by release of calcium from ryanodine-sensitive stores has been hypothesized to underlie the development of a depolarizing after potential (DAP) in the MNCs (Li and Hatton, 1997).

A variety of studies using models of neural plasticity have demonstrated synergistic interactions between mGluRs and NMDA receptors attributed to opening of voltage-gated calcium channels (Wang et al., 1997) and activation of protein kinases (Bortolotto and Collingridge, 1998; Fukunaga et al., 1992). Examination of the interactions between osmotic and metabotropic receptors using agonists specific for types I, II and III subclasses of mGluRs supported the negative effects of osmotic stimulation on calcium increases due to type I mGluR activation (DHPG). Osmotic stimulation had only small effects on types II and III mGluR stimulation. These receptors are largely coupled to G proteins that inhibit cAMP production (Pin and Duvoisin, 1995), and therefore do not directly influence the mobilization of intracellular calcium. Nevertheless, the rapid decline of intracellular calcium in the MNCs after stimulation with the type II agonist, DCG-IV or the type III agonist, AP-4 suggested that these receptors may indirectly control MNC calcium. The high expression of mGluR3 mRNA and mGluR2/3 protein in the SON (Al Ghoul et al., 1998) suggest that this subtype, in particular, may participate in the regulation of MNC function. This possibility was supported by the enhanced glutamate-induced calcium increase in the presence of the broad spectrum mGluR antagonist, (+)MCPG. This suggests the possibility of strong intrinsic pre- or postsynaptic inhibition by mGluRs in these cultures.

4.3. Pathological implications

In addition to a role in water balance regulation in the MNCs, the strong synergistic relationship between osmotic and NMDA receptor stimulation is likely to have widespread implications under a variety of pathological conditions. We have previous observed that a hyperosmotic stress induced by water deprivation can induce c-Jun-N-terminal protein kinases (JNK) in a variety of brain regions (Meeker and Fernandes, 2000). These results were confirmed in vitro where NMDA receptor activation and osmotic stimulation were both shown to be potent stimuli for JNK upregulation. Osmotic contributions to NMDA receptor and JNK activation could be important in clinical conditions where dehydration and high plasma osmolality may be present. For example recent studies of the relationship between plasma osmolality and the outcome of acute stroke observed that plasma osmo-

lalities above 296 mosM/kg were significantly associated with mortality (Bhalla et al., 2000). In animal models, excessive dehydration with hypertonic mannitol has been shown to increase brain water content and induce midline shift after unilateral middle cerebral artery occlusion (Paczynski et al., 2000). These deleterious effects could be predicted from the current findings. On the other hand, cellular dehydration has been reported to suppress generalized seizure activity (Andrew et al., 1989) and hypertonic mannitol may reduce necrotic edema following severe kainic acid-induced seizures (Nelson and Olson, 1987). Thus, the effects of osmotic stimuli are likely to be complex depending on the cell types and specific brain regions. For example, in addition to mechanoreceptors sensitive to the effects of hyperosmotic stimulation, many cells also possess osmotically sensitive, stretch-activated mechanoreceptors (osmoreceptors) (McCarty and O'Neil, 1992; Sachs, 1991). Such mechanoreceptors may be responsible for osmotic effects on astrocytes (Bres et al., 2000; Hussy et al., 2000). The way in which various cells respond to an osmotic stress will depend on the nature of the mechanoreceptors, as well as numerous other physiological and pathophysiological inputs. More work is needed to determine how osmotic changes influence glutamate receptor and signal transduction pathways in various neural tissues under normal and pathological conditions.

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