The Expression of the PDZ Protein MALS-1/Velis Is Regulated by Calcium and Calcineurin in Cerebellar Granule Cells*

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Activity-dependent gene expression is thought to be important in shaping neuronal development and in modifying the protein content of neurons. Ca\(^{2+}\) entry into neurons appears to be one of the key effectors of activity-dependent gene expression. Among the possible downstream targets of calcium, the protein phosphatase calcineurin represents a prime candidate. We hereby report that in cultured cerebellar granule cells the activation of the Ca\(^{2+}\)/calcineurin pathway via either voltage- or ligand-operated Ca\(^{2+}\) channels regulates MALS-1 and MALS-2 expression at the transcriptional level. These proteins are integral parts of the post-synaptic density and are also involved in receptor trafficking. MALS regulation is not at the level of mRNA stability and does not require de novo protein synthesis, thereby suggesting a direct pathway. These data suggest that Ca\(^{2+}\) entry by means of calcineurin is capable of controlling the structure of the post-synaptic density by controlling the expression of key components at the transcriptional level.

Activity-dependent gene expression is thought to play an important role in shaping neuronal development and in selecting protein content of neurons in general (1). Among the crucial mediators of activity-dependent gene expression is the activation of voltage- and ligand-gated Ca\(^{2+}\) channels and the subsequent influx of calcium (1). This can lead to pivotal changes to neuronal behavior such as long-term potentiation and progression in development (2). For example, in cultured cerebellar granule cells, it is established that a small rise in intracellular Ca\(^{2+}\) concentrations (usually evoked by depolarizing concentrations of KCl) leads to cell survival, probably because of increased gene transcription (3, 4). This stimulation is thought to mimic the connections made by developing cerebellar granule cells with climbing mossy fibers (4, 5). Ca\(^{2+}\)-dependent gene expression has been shown to follow several signaling pathways (1, 6), but our knowledge on the transcriptional mediators that eventually are directly affected is restricted. Most of the interest in neurons has been directed toward the activation of Ca\(^{2+}\)/calmodulin-dependent kinases and their downstream effectors (7). However, in T-lymphocytes, in cardiac and skeletal muscle cells, one of the most important molecular switches for Ca\(^{2+}\)-dependent gene transcription appears to be the serine-threonine phosphatase calcineurin (8–11). Calcineurin can dephosphorylate transcription factors (e.g. NFAT) or can contrast the effects of Ca\(^{2+}\)/calmodulin-dependent kinases on cAMP-response element-binding protein (CREB) by controlling the activity of protein phosphatase 1 (12, 13). In neurons, the role of calcineurin has not been investigated thoroughly. Recently, we and others have shown that genes that participate in Ca\(^{2+}\) signaling (inositol 1,4,5-trisphosphate receptors, calcium ATPase pumps, and Na\(^{+}/Ca\(^{2+}\) exchangers) are tightly controlled at the transcriptional level by calcineurin (14–18) and that glycogen synthase kinase 3 appears to contrast this response (15).

Alongside components of the calcium signaling machinery, it is probable that other key cellular functions are affected by Ca\(^{2+}\) influx at the transcriptional level. In neurons, the post-synaptic density (PSD) is a complex-scaffolding structure that plays a pivotal role in spatially organizing receptors and signaling effectors, thus leading to a broad functional organization of the synapse (20). Because the PSD is a dynamic structure, it can be hypothesized that it could be modified to support changes in the neuronal activity that may occur. The family of MALS proteins (MALS-1–3, also known as mLin-7 or Velis) has emerged as a key component of the post-synaptic density (21, 22) and is thought to play a role in the trafficking and docking of plasma membrane receptors, e.g. to the NR2B subunit of the glutamate NMDA receptor (23), and play a role in neurotransmitter vesicle exocytosis (24).

Because in cerebellar granule cells, depolarization by mimicking synaptic activity induces numerous changes that should influence the post-synaptic density (e.g. switches in glutamate receptor subtypes) (25, 26), we have investigated whether such changes could be paralleled by changes in MALS levels. This would represent a way for extracellular stimuli to induce maturation and plasticity of synapses. We now report that MALS-1 and MALS-2 are tightly controlled at the transcriptional level by Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels when cells are chronically depolarized. To strengthen this result, we have also shown that the physiological stimulus, i.e. activation of glutamate receptors, is capable of controlling MALS protein levels to the same extent. Calcineurin is the downstream effector of this effect because FK506 and cyclosporin A abolish Ca\(^{2+}\) influx-induced expression of MALS isofoms. Furthermore, the transcriptional activation of MALS-1 is rapid and does not require de novo protein synthesis, thereby suggesting that accumula-

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The abbreviations used are: CREB, cAMP-response element-binding protein; PSD, post-synaptic density; RT, reverse transcription; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MAPK, mitogen-activated protein kinase; AMPA, \(\alpha\)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid.
tion of this protein might precede other compositional changes at the PSD.

**MATERIALS AND METHODS**

**Cell Culture**—Granule cells were dissociated from the cerebella of 7-day-old Wistar rats as described previously (3, 14) and plated in Dulbecco’s modified Eagle’s medium HEPES modification containing 1.8 mM CaCl<sub>2</sub> supplemented with 10% fetal calf serum, 100 µg/ml gentamicin, 100 µg/ml pyruvate on poly-l-lysine-treated plates at a density of 2-3 x 10<sup>6</sup> cells/cm<sup>2</sup>. Twenty-four hours after plating, culture medium was removed and fresh medium was added. This medium included arabinoside cytosine (10 µM) and any drug or treatment. The 6-h experiment with NMDA was conducted in Mg<sup>2+</sup>-free Locke’s solution.

**Isolation of RNA and Reverse Transcription (RT)-PCR**—Total RNA was prepared from granule cells by using the TRIzol reagent according to manufacturer’s instructions (Invitrogen) and was reverse-transcribed by using an oligo(dT) primer (first strand cDNA synthesis kit, Qiagen, Crawley, United Kingdom) according to the manufacturer’s protocol.

The following oligonucleotides were used as primers for PCR: MALS-1 forward, 5'-gaatccgagatgg-3', and reverse, 5'-tccttagcagcttgagaag-3'; MALS-2 forward, 5'-gtgctcgccggtg-3'; and reverse, 5'-atctctccagcagc-3'; and actin forward, 5'-aagaggtcagcactcctcag-3', and reverse, 5'-ctttcattggagtctgtcagttcc-3'.

The conditions for the PCR reactions were as suggested from Promega for the Taq polymerase. PCR products were separated on 1.8% agarose gels. All of the reactions resulted in a single band with the exception of the MALS-1 experiments where RNA was extracted from tissues and where two bands corresponding to two splicing variants were amplified.

**RESULTS AND DISCUSSION**

MALS-1 appears to be the most expressed isoform of the MALS family of genes in the adult cerebellum (22), and we set out to investigate whether in cerebellar granule cells its mRNA levels might have been modulated by chronic depolarization. Cells grown under depolarizing conditions for 3 days (25 or 40 mM) appeared to express significantly higher MALS-1 mRNA levels compared with cells grown in the presence of 5.3 or 10 mM KC1 (Fig. 1A). Although the PCR primers were designed to be specific for the MALS-1 isoform, the similarity between MALS genes generated the possibility that MALS-2 might have been amplified as well by this set of primers. To rule out this hypothesis, the amplified PCR product was digested with XhoI, which should selectively cut the MALS-2 product. This procedure did not result in any apparent digestion fragments, confirming the sole presence of MALS-1. Furthermore, the PCR product was sequenced and shown to be MALS-1. The use of a primer located on the 3'-untranslated region revealed that the MALS-1 splice variant present in cerebellar granule cells corresponds to Lin-7Bb (data not shown) (27).

We then examined whether the presence of extracellular calcium was important to influence the up-regulation of MALS-1 gene and whether the effect could be observed after shorter treatments. This is important because in cerebellar granule cells depolarization via Ca<sup>2+</sup> influx mediates the survival of cells, and although substantial cell death occurs only after 3 days in vitro (data not shown) (3, 4),<sup>1</sup> these results could still be indicative of this situation. Indeed, 6 h after the addition of 25 mM KCl where no cell death was observed, the up-regulation was detectable and the addition of EGTA to the extracellular medium prevented this change, although no differences were observed for actin (Fig. 1B). Therefore, it would appear that cell survival does not play a role in this observation.

To further characterize the possible mechanism by which Ca<sup>2+</sup> controls MALS-1 up-regulation, cerebellar granule cells were cultured for 6 h in the presence of 25 mM KCl and in the presence of either L-type Ca<sup>2+</sup> channel blockers or NMDA receptor antagonists. Nifedipine (1 µM) and diltiazem (1 µM) (data not shown) blocked the up-regulation induced by depolarization, whereas MK801 was unable to substantially affect MALS-1 mRNA (Fig. 2A).

We next investigated whether MALS-1 mRNA could be regulated by calcineurin. Both FK506 and cyclosporin A, structurally different inhibitors of calcineurin (28), were able to selectively down-regulate the expression of MALS-1 (Fig. 2B),

**D. Kramer and A. A. Genazzani, unpublished results.**
suggesting that Ca\textsuperscript{2+} influx activates this phosphatase, which in turn is able to trigger the transcriptional machinery required for MALS-1. It has recently been reported that the immediate early gene Homer 1a, which also cooperates in shaping the post-synaptic density (29), is up-regulated by an NMDA- and Ca\textsuperscript{2+}/H\textsubscript{11001}-dependent mechanism in these cultures (30). This effect appeared to be independent of L-type Ca\textsuperscript{2+}/H\textsubscript{11001}-channels but appears to be mediated by the activation of a mitogen-activated protein kinase cascade (30). To investigate whether this pathway could be responsible for MALS-1 up-regulation as well, we treated cells with the specific MEK inhibitor PD98059 (30 M), which is able to revert the Homer1a up-regulation (30). Surprisingly, this drug was unable to influence MALS-1 expression induced by depolarization. This would suggest that neurons are capable of decoding differently the Ca\textsuperscript{2+} stimuli and can thereby alter the post-synaptic density accordingly. To support this finding, we attempted to induce Homer 1a expression either by depolarization or glutamate receptor stimulation, but no signal was detected. This might suggest that the pathway involved in Homer 1a regulation is developmentally regulated and is not present in the early stages of the culture.

As a further control to strengthen the reliability of our model, we performed a RT-PCR with specific primers for NCX2, the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger isoform that is selectively controlled by calcineurin (18). As expected, cells grown in 25 mM KCl expressed lower levels of this isoform (Fig. 2B). Surprisingly, our data suggest that the MAPK-signaling cascade might be involved in this down-regulation. It has been previously shown that the NCX2 down-regulation occurs in a fast manner with significant changes observable after 1 h (18). When a time course of MALS-1 up-regulation was performed, no changes at the mRNA level were observed after 1 h, suggesting that MALS-1 is not an immediate early gene (Fig. 2C), whereas substantial up-regulation was observed after 6 h. To determine a relative level of change of MALS-1 expression according to the different treatments, we performed a densitometric analysis of the bands corresponding to MALS-1 changes from four separate experiments. Results are normalized for the respective actin controls and expressed as the percentage of the K5 samples. *, p < 0.001, Student’s t test.

![Fig. 2. Characterization of MALS-1 expression by RT-PCR.](image)

Control of MALS-1 Expression in Neurons
Control of MALS-1 Expression in Neurons

**Fig. 3.** MALS-1 is developmentally regulated and its protein levels are controlled by calcineurin. A, Western blot of cell extracts treated for different times (2, 4, and 6 days) with 25 mM KCl in the presence or absence of FK506 (FK) (1 μM) were probed with an anti-MALS antibody and then stripped and reprobed with an anti-actin antibody. Membrane fractions were used to probe for PSD-95 changes. Control cells (5) were not supplemented with KCl. B, RT-PCR with MALS-1-specific primers of cerebellar and cortical total RNA extracted from 1-day-old (1), 8-day-old (8), and adult (A) rats. DIV, days in vitro.

Predicted, MALS-1 underwent a developmental up-regulation, and its expression was highest in extracts from cells grown in K25.

Protein expression was virtually abolished in cells grown in non-depolarizing KCl or in depolarized cells grown in the presence of 1 μM FK506 (Fig. 3A). Because we have previously shown that growing cells in the presence of FK506 does not affect neuronal survival (14), this observation cannot be explained by a selective neuronal loss. Furthermore, it has been shown that the cells surviving in 5 mM KCl are morphologically indistinguishable from cells grown under depolarizing conditions (16). The changes are protein-specific, because it has been previously shown that control proteins such as the ryanodine receptor or isoforms of the plasma membrane Ca²⁺-ATPase pumps or of the Na⁺/Ca²⁺ exchanger, which are not transcriptionally regulated by calcineurin, show identical protein bands independently of treatments (16, 17). Furthermore, no changes in expression were observed when Western blots were probed with antibodies against the post-synaptic protein PSD-95 or with actin (Fig. 3A), suggesting that MALS-1 changes are specific and are not attributed to a broad rearrangement of the synapse regardless of treatments. Surprisingly, the band corresponding to the other two isoforms appeared to be regulated in similar fashion by Ca²⁺ and calcineurin, although the expression of these isoforms appeared to be highest after 3 days in culture and appeared to decline thereafter. Although the antibody might not necessarily recognize the three proteins to the same extent, it is apparent that relative MALS-1 expression is increased during development compared with the other two isoforms. The relative change in the proportion of MALS-1 present is in accord with previous protein analysis in whole brain homogenates during development (21). To further confirm this finding, RT-PCR analysis of RNA extracted from cerebellum or cortex during different stages of development was performed. As expected, MALS-1 signal was increased significantly through development with the lowest signal observed in 1-day-old rats and the highest signal observed in adults (Fig. 3B). This trend is highly suggestive of an increased expression with increased neuronal activity and parallels the data in the cell culture.

Because the main input to cerebellar granule cells is represented by glutamatergic mossy fibers, it could be envisaged that the physiological synaptic stimulus in vivo would be represented by glutamate. To investigate this in our model, we treated cells for 6 h in the presence of glutamate (100 μM), NMDA (30 μM), AMPA (100 μM), FK506 (1 μM), and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (30 μM). DNQX was applied 1 h before stimulation. Glut, glutamate. B, cells were incubated for the stated times in the absence or presence of NMDA (50 μM).

**Fig. 4.** Effect of synaptic stimuli on MALS-1 expression. A, cells were incubated for 6 h in magnesium-free solution in the presence of glutamate (100 μM), NMDA (30 μM), AMPA (100 μM), FK506 (1 μM), and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (30 μM). DNQX was applied 1 h before stimulation. Glut, glutamate. B, cells were incubated for the stated times in the absence or presence of NMDA (50 μM).

Although it is possible that a small amount of Ca²⁺ will flow through the AMPA receptor channel itself, it is probable that most of the Ca²⁺ influx induced by AMPA will be mediated by voltage-operated Ca²⁺-channels as suggested previously (31, 32). NMDA receptor activation resulted in a similar time course compared with depolarization (Fig. 4B), suggesting that these two protocols of stimulation work through a similar mechanism.

To further confirm the role of calcineurin, proteins were extracted from cells grown in the presence of either FK506 or cyclosporin A for 2 days (Fig. 5A). Both drugs induced a significant reduction of the intensity of the bands corresponding to the MAL3 isoforms, paralleling observations obtained by RT-PCR. Furthermore, the blockade of L-type Ca²⁺ channels also abolished MALS expression. Because it appears that both the band specific for MALS-1 and the band representing MALS-2 and MALS-3 are sensitive to calcineurin inhibitors, we performed an RT-PCR experiment with primers specific for the MALS-2 isoform. Surprisingly, this isoform encoded by a different gene on a different chromosome appears to be controlled by calcineurin at the DNA level as well (Fig. 5B). Nonetheless, it is interesting to note that other factors might be involved in
the control of these two proteins because MALS-1 appears to increase during development, whereas MALS-2 undergoes a decrease (Fig. 3A). Calcineurin could either participate in the control of MALS-1 transcription or could affect the stability of MALS mRNA. Although most effects attributable to calcineurin occur at the transcriptional level, mRNA stability has been shown to be affected in particular cell types (33). To test which of these two modes of action accounts for the change in MALS-1 expression, we induced the expression of mRNA for 6 h in the presence or absence of 25 mM KCl and, thereafter, we added calcineurin inhibitors and the RNA polymerase inhibitor actinomycin D to monitor the decay in RNA. When normalized for cDNA amounts, MALS-1 signal decreased with time over a 6-h period. The decay of MALS-1 signal was not significantly different between control cultures and cultures grown in the presence of FK506 or cyclosporin A, thereby making it unlikely that mRNA stability is the major root of modulation (Fig. 6A).

As a control, changes in actin mRNA were also investigated and a similar trend was observed with a decrease regardless of treatment (data not shown). Calcineurin could either directly influence one or more of the transcription factors responsible for MALS-1 up-regulation. It might influence its transcription indirectly by positively or negatively modulating the expression of a transcription factor not present under basal conditions, or it might influence the activity of a transcription factor not present under basal conditions but whose expression is dependent on Ca²⁺ influx. To determine which of these possibilities is most probable, we performed experiments in the presence of cycloheximide, thereby blocking protein synthesis. In the presence of cycloheximide and 25 mM KCl, the amount of mRNA encoding for MALS-1 increased significantly (Fig. 6B). Therefore, it would appear that the machinery responsible for activation of MALS-1 transcription is within neurons under basal conditions, and the effect by calcineurin is probably a dephosphorylation of a transcription factor such as NFAT or the control of MEF2 activity (8, 34). Furthermore, preliminary evidence from our laboratory indicates that these transcription factors are present either at the protein level⁴ or at the RNA level,⁴ respectively. Nonetheless, at present it cannot be excluded that the ubiquitous transcription factor CREB might be involved in the control of MALS proteins. It is known that

³ L. Fresu and A. A. Genazzani, unpublished results.
⁴ T. Ellis, T. Lori, and A. A. Genazzani, unpublished results.

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**Fig. 5.** MALS-1 and MALS-2 are regulated in similar fashion by calcium and calcineurin after 3 days in culture. A, Western blotting of cellular extracts from neurons grown in complete medium in the presence or absence of depolarizing concentrations of KCl. Cells grown in the depolarizing medium were also grown in the presence or absence of FK506 (FK) (1 μM), cyclosporin A (CsA) (1 μM), or nifedipine (Nif) (1 μM). The two panels represent the same experiment at different exposure times, because the smaller molecular weight band is substantially more expressed at this developmental stage (see Fig. 3A). An actin antibody was used to probe for equal loading and transfer. PSD-95 levels were analyzed on the membrane fraction. B, RT-PCR analysis of MALS-1 and MALS-2 gene expression at the same time point as in part A. DIV, days in vitro.

**Fig. 6.** MALS-1 expression is not secondary to mRNA stability changes and does not require protein synthesis. A, cells grown for 6 h in the presence or absence of 25 mM KCl were subsequently treated with actinomycin D (ActD) in the presence or absence of FK506 or cyclosporin A for the indicated time points. B, cells were incubated for 6 h in complete medium supplemented with 25 mM KCl in the presence or absence of FK506, cyclosporin A, or the protein synthesis inhibitor cycloheximide (CHX) (5 μg/ml).
Calcineurin can modulate the activity of the inhibitory peptide for protein phosphatase 1, and therefore, it could indirectly regulate the phosphorylation state of CREB (12). Interestingly, initial bioinformatics investigation of the MALS-1 gene in the human genome revealed that NFAT, MEF2, and CREB binding sites exist in the putative promoter regions of both MALS-1 and MALS-2 genes.

In conclusion, we have shown that MALS-1 and MALS-2 are regulated at the transcriptional level by Ca\(^{2+}\)/CaM influx feeding onto the activation of the Ca\(^{2+}\)/CaM phosphatase calcineurin. The activation of both voltage-operated Ca\(^{2+}\) channels or more physiologically of glutamate receptors is sufficient to elicit an up-regulation. Nonetheless, MALS-1 expression appears to be more long lasting compared with MALS-2. An explanation for this could be that other transcription factors mediate the developmental profile of the expression of MALS isoforms and that calcineurin plays a permissive role. Although the full potential of MALS proteins is at present unknown, it has been shown to play a role in vesicle exocytosis (24), protein trafficking (23), and PSD assembly (21). Therefore, it perhaps comes as no surprise that in the cerebellum its expression is mediated by synaptic activity. In other words, cerebellar neurons will react to the formation of new synapses by increasing MALS-1 expression and consequently increasing the capacity of the neuron to deal with new inputs. It is well documented that calcineurin activation requires protracted low elevations of calcium, which in vitro can be delivered by mild depolarization (19) or glutamate receptor activation; therefore, it can be hypothesized that in vivo the inputs will generate such stimuli to increase MALS expression. Recently, it has been shown that Homer 1a, a natural dominant negative of the PSD, can be up-regulated as well in these cells, albeit only by NMDA activation (30). Therefore, it would appear that Ca\(^{2+}\) influx in the post-synaptic terminal is capable of shaping the future architecture of the post-synaptic density by altering transcription.

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