Chemical Stimulation of Synaptosomes Modulates α-Ca^{2+}/Calmodulin-Dependent Protein Kinase II mRNA Association to Polysomes

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The presence of specific mRNAs in dendrites and at synapses is well established, but a direct and reliable demonstration that they are associated with polysomes is still missing. To address this point we analyzed the polysomal association of the mRNAs for the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II (α-CaMKII), for type 1 inositol 1,4,5-trisphosphate receptor (InsP3R1) and for the activity-regulated cytoskeleton-associated protein (Arc) in a synaptosomal preparation devoid of contaminating material from neuronal and glial perikarya. We show that a fraction of α-CaMKII, InsP3R1, and Arc mRNAs present in synaptosomes is indeed associated with polysomes. Moreover, we show that polysomal association of α-CaMKII mRNA, but not InsP3R1 and Arc mRNAs, increases with depolarization of the synaptosomal membrane. Finally, we show that the synthesis of α-CaMKII protein increases with stimulation. Dendritic mRNA recruitment onto polysomes in response to synaptic stimulation might represent one of the mechanisms underlying the processes of learning and memory.

Key words: α-CaMKII; synaptosomes; chemical stimulation of synaptosomes; translation regulation; polysome/mRNA distribution of mRNA; synaptic plasticity

Local protein synthesis in dendrites is supported by the finding of ribosomes, tRNAs, translation factors, and specific mRNAs at postsynaptic sites (Steward and Levy, 1982; Chicurel et al., 1993; Knowles et al., 1996). It has been suggested that dendritic polyribosomes might synthesize some of the protein constituents of the postsynaptic membrane specialization (Steward and Falk 1986; Palacios-Pru et al., 1988), thus being implicated in long-term changes after synaptic activation (Weiler and Greenough, 1993; Martin et al., 1997; Morris, 1997; Schuman, 1997). Dendritic localization has been demonstrated for several mRNAs (Steward, 1997; Kuhl and Skehel, 1998), and the mechanisms underlying specific mRNA transport are beginning to be studied (Mayford et al., 1996; Muslimov et al., 1997; Tongiorgi and Cattaneo, 1997; Wu et al., 1998). Although several lines of evidence support the notion that the protein synthesis machinery localized in dendrites and at the postsynapses is functional (Crino and Eberwine, 1996; Huang, 1999), direct and clear evidence that specific mRNAs are associated with polysomes within dendrites has not yet been reported.

The expression of the α-subunit of Ca^{2+}/calmodulin-dependent protein kinase II (α-CaMKII) mRNA in dendrites is particularly interesting, because there is strong evidence that this protein plays a role in certain forms of synaptic plasticity, such as long-term potentiation (Silva et al., 1992; Ouyang et al., 1997). Moreover, the mRNA for the α subunit of CaMKII is also localized in dendrites (Burgin et al., 1990). To investigate whether synaptic mRNAs were actively translated, we analyzed the polysomal association of α-CaMKII, type 1 inositol 1,4,5-trisphosphate receptor (InsP3R1), and activity-regulated cytoskeleton-associated protein (Arc) mRNAs in a synaptosomal preparation devoid of contaminating material from the perikarya of neuronal and glial cells and the change in mRNA translation and protein synthesis after synaptic stimulation.

MATERIALS AND METHODS

Isolation of synaptosomes from mouse brain. Animal care was conducted in conformity with institutional guidelines that are in compliance with national (Decreto Legge N116, Gazzetta Ufficiale, suppl 40, 18-2-1992) and international laws and policies (European Community Council Directive 86/609, Official Journal, Law 358, 1, December 12, 1987; National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, US National Research Council, 1996). Three- to 4-week-old C57BL/6 mice were killed by cervical dislocation. All of the following steps were performed at 4°C. Brains were dissected and homogenized in isotonic sucrose (320 mM), pH 7.4, 1 mM EDTA, 0.25 mM dithiothreitol, and 30 U/ml RNase inhibitor (Amersham Pharmacia Biotech, Uppsala, Sweden). The homogenate was centrifuged at 1000 $\times$ *g* for 10 min. The

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supernatant was loaded on a Sucrose-Percoll discontinuous gradient and centrifuged at 32,000 × *g* for 5 min (Dunkley et al., 1986). Crude synaptosomes were recovered, washed in PBS, and centrifuged at 12,000 × *g* for 4 min. The supernatant was carefully removed, and the synaptosome containing pellet was resuspended in 50% OptiPrep (Accurate Chemical, Westbury, NY), loaded on an OptiPrep discontinuous floatation gradient (9, 12.5, 15, 25, and 35%), and centrifuged at 10,000 × *g* for 20 min (modified from the method of Kiebler et al., 1999). The recovered synaptosome-containing band (15–25% interface) was centrifuged at 30,000 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in the appropriate buffer.

Synaptosome stimulation. For polysome analysis, synaptosomes were resuspended in 100 μl of 10 mM Tris, pH 7.5, 2.2 mM $CaCl_2$, 0.5 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4 mM $NaHCO_3$, and 80 mM NaCl and stimulated by addition of 50 mM KCl or 300 μM glutamate plus 10 μM glycine, followed by 5 min incubation at 37°C. For protein synthesis analysis, stimulation was performed as above, except that incubation was performed for 45 min at 37°C in the presence 30 μCi of Pro-mix L-^{35}S *in vitro* cell labeling mix (Amersham Pharmacia Biotech), with 100 μg/ml chloramphenicol and 15 μl of protease inhibitor mixture (Sigma, St. Louis, MO). Synaptosomes were then frozen in liquid nitrogen and stored at −70°C.

Electron microscopy. The synaptosome-containing fraction from the discontinuous gradient was diluted in two volumes of PBS twice and centrifuged at 10,000 rpm for 10 min. The pellet was fixed in 2.5% glutaraldehyde, 1.24 mM $CaCl_2$, 1.24 mM $MgCl_2$, and 70 mM KCl in 100 mM cacodylic acid, embedded and processed for electron microscopy as described (Parton et al., 1992), and examined in a Zeiss (Thornwood, NY) EM10 microscope.

Protein and RNA extraction from synaptosomes. General procedures for protein and RNA preparation and analysis followed standard laboratory manuals. Proteins from total brain and from synaptosomes were resuspended in Laemli buffer, boiled, analyzed by polyacrylamide gel electrophoresis, and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The detection was performed using the SuperSignal chemioluminescent substrate (Pierce, Rockford, IL).

RNA extraction from total brain, synaptosomes, and gradient fractions (see below) was performed out by the proteinase K-SDS method, phenol-chloroform extraction, and ethanol precipitation in the presence, when necessary, of 10 μg of glycogen as a carrier. For Northern analysis, RNA was fractionated by formaldehyde-agarose gel electrophoresis and transferred on a Gene Screen Plus membrane (New England Nuclear Life Science Products, Boston, MA); hybridization was performed essentially according to the manufacturer's instructions. Radioactive probes were prepared by random priming. Hybridization filters were exposed to x-ray films and quantitatively analyzed by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Antibodies. Synaptophysin and α-CaMKII monoclonal antibodies were purchased from Roche Molecular Biochemicals (Mannheim, Germany); MAP2 monoclonal antibody was from Sigma; and PSD-95 and transglutaminase (TGase) antibodies were kindly provided by David Bredt (Department of Physiology, University of California, San Francisco, CA) and Mauro Piacentini (Department of Biology, University of Rome "Tor Vergata"), respectively.

Polysome/ribonucleoprotein distribution of mRNAs. Extract preparation, sucrose gradient sedimentation of polysomes, and analysis of the polysome/messenger ribonucleoprotein (mRNP) distribution of mRNAs were performed as described (Meyuhas et al., 1996). Briefly, total brain homogenization and synaptosome lysis were performed in lysis buffer (10 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 1 mM dithiothreitol, 30 U/ml RNase inhibitor (Amersham Pharmacia Biotech), and 100 μg/ml cycloheximide). After 5 min of incubation on ice the lysates were centrifuged for 8 min at 12,000 × *g* at 4°C. The supernatants were frozen in liquid nitrogen and stored at −70°C or immediately sedimented in a 5–70% (w/v) sucrose gradient by centrifugation for 135 min at 37,000 rpm in a Beckman Instruments (Palo Alto, CA) SW41 rotor. Each gradient was collected in 10 fractions while monitoring the absorbance at 254 nm. RNA extracted from gradient fractions was analyzed by Northern blot hybridization or by RT-PCR.

RT-PCR amplification. RNA samples extracted from synaptosomes were DNase-treated, and an aliquot of each sample was reverse-transcribed into cDNA by the random hexanucleotide technique using 100 U of Moloney murine leukemia virus reverse transcriptase ($RNaseH^-$; Life Technologies Italia, Milan, Italy) according to the manufacturer. For quantitative RT-PCR analysis of RNA extracted from sucrose gradient fractions, an equal amount (10 pg) of an internal control RNA was added to each fraction before reverse transcription. This RNA was obtained by *in vitro* transcription (Ambion, Austin, TX) of the *Xenopus* ribosomal protein L27A sequence (formerly L22, GenBank accession number X64207) and detected with specific oligonucleotides. An aliquot of RT reaction was PCR-amplified in a final volume of 50 μl by using 20 pmol of each primer, a 200 μM concentration of each dNTP, and 0.5 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). When quantitative amplification was required, the amount of template and the number of amplification cycles were preliminarily optimized for each PCR reaction to avoid conditions of saturation. For radioactive PCR, dCTP was reduced to 10 μM, and 0.2 μCi of [α-^{32}P]dCTP (Amersham Pharmacia Biotech; 3000 Ci/mmol) was added. Radioactive gels were exposed to x-ray films and quantitatively analyzed by a PhosphorImager.

RESULTS

To study mRNA translation at the synapses, we used an improved method for preparing synaptosomes from mouse neocortex in such a way that the preparation was enriched in synaptic terminals and essentially free of contamination from perikarya of neuronal and glial cells. Because our aim was to study dendritic mRNA translation, the presence of presynaptic terminals (axonal), which, for the most part, lack mRNAs, was not consequential. The procedure, based on two discontinuous gradient centrifugations (Dunkley et al., 1986; Kiebler et al., 1999), is described in Materials and Methods.

To set up and optimize the preparation procedure, the quality and purity of synaptosomes were monitored by electron microscopy, Western analysis, and RT-PCR. Electron microscopy analysis, in which presynaptic particles are defined by the presence of vescicles and the presence of postsynaptic densities and polysomes defines the postsynaptic particles, has been useful in preliminary experiments to verify the good preservation of synaptosomes (see example in Fig. 1*a*). However electron microscopy is not a suitable method to evaluate preparation purity, because not all structures observed can be clearly identified. For instance a synaptosome cannot be unequivocally recognized if the section does not cut through both presynaptic and postsynaptic portions. The purity of the preparations was better ascertained by Western analysis with antibodies specific for presynaptic, postsynaptic, and cell body proteins. Figure 1*b* shows that the presynaptic protein synaptophysin, the small dendritic MAP2 protein isoforms, and the postsynaptic protein PSD-95 are highly enriched in these synaptosomal preparations. On the contrary, TGase, a protein present only in the cell body, is found in total brain extract but not in synaptosomes. Moreover, the presence of small amounts of dendritic and cell body mRNAs was analyzed by radioactive RT-PCR with specific primers. Figure 1*c* shows that α-tubulin mRNA, as well as the astrocyte-specific glial fibrillary acidic protein (GFAP) mRNA, are not detected in our synaptosome preparations, whereas well established (Steward, 1997) dendritic mRNAs, such as those for Arc, α-CaMKII, and InsP3R1, are present. These results indicate that our synaptic preparations are free from cell body contamination and thus very suitable for studying synaptic mRNA translation.

A reliable way to assess the mRNA translational efficiency is to analyze its association with polysomes (Meyuhas et al., 1996). This is performed by sucrose gradient fractionation of a cytoplasmic extract followed by Northern blot analysis of RNA extracted from the different fractions of the gradient. We first analyzed the cytoplasmic extract from total brain. Figure 2*a* shows the typical polysomal profile, and Figure 2*b* shows the Northern analysis of the 10 gradient fractions hybridized with a probe for α-CaMKII

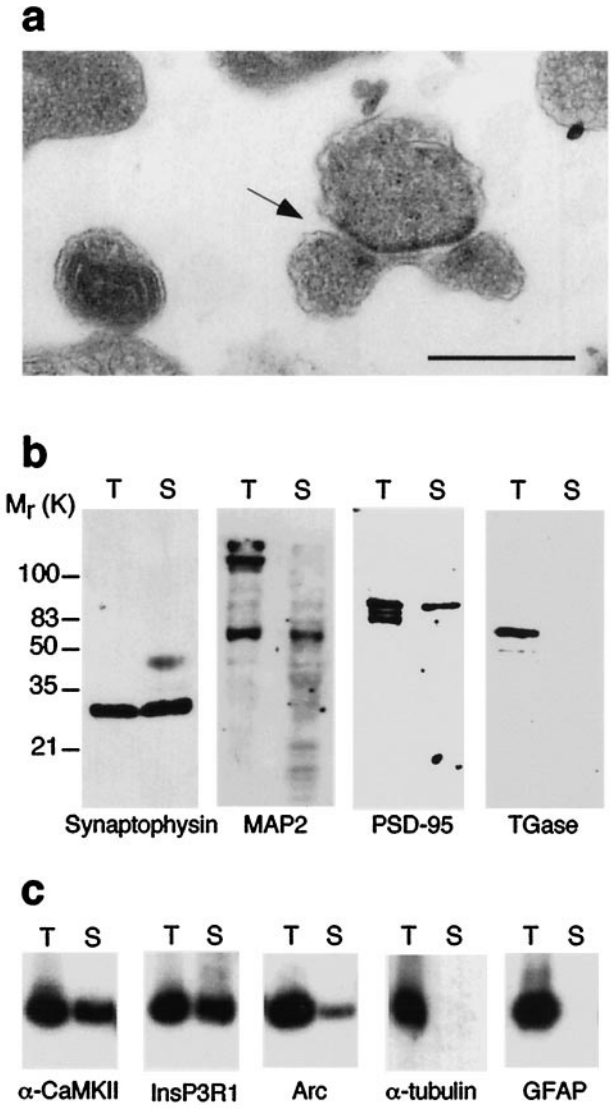

Figure 1. Quality assessment of synaptosome preparations. *a*, Electron micrograph showing a synaptosome with preserved presynaptic and postsynaptic elements. Scale bar, 0.5 μm. *b*, Western blot analysis of proteins prepared from synaptosomes (*S*) and from total brain (*T*), with antibodies specific for presynaptic and postsynaptic proteins (*Synaptophysin, MAP2, PSD-95*) and for the nonsynaptic TGase protein. *c*, Radioactive RT-PCR has been used as a very sensitive method to analyze specific mRNAs in synaptosomes (*S*) and in total brain (*T*). Specific primers have been used for PCR detection of the dendritic α-CaMKII, InsP3R1, and ARC mRNAs, the cell body α-tubulin mRNA, and the glial GFAP mRNA.

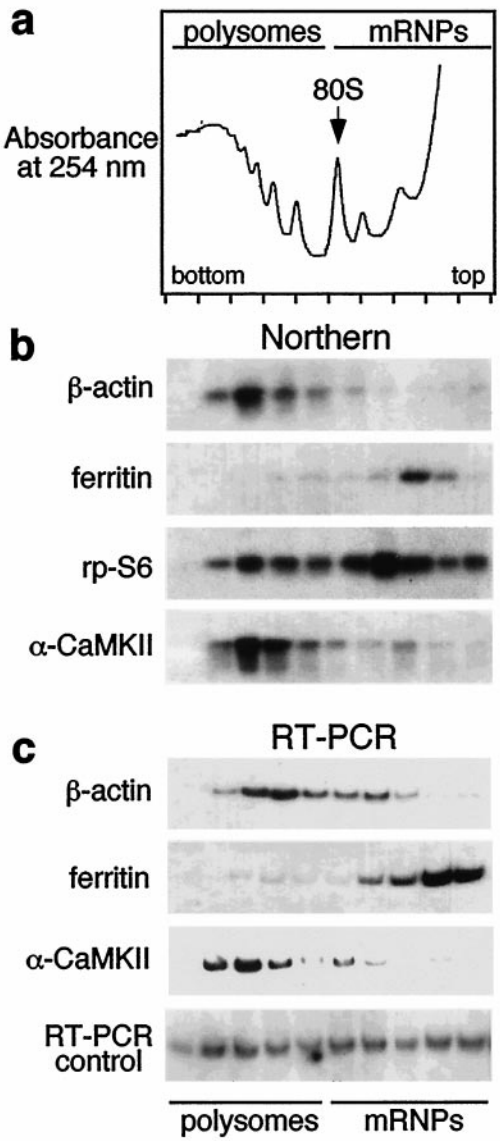

Figure 2. Polysome and mRNP distribution of mRNAs in total brain. Total brain cytoplasmic extract was fractionated by sucrose gradient centrifugation. Each gradient was collected in 10 fractions while monitoring the absorbance at 254 nm. *a*, Absorbance profile of a sucrose gradient showing a typical polysomal pattern. *b*, Northern analysis of gradient fractions with probes specific for β-actin, ferritin, rp-S6, and α-CaMKII mRNAs. *c*, Quantitative radioactive RT-PCR analysis of gradient fractions with primers specific for β-actin, ferritin, and α-CaMKII mRNAs and for a synthetic RNA previously added in equal amounts to all fractions as an internal control. The amount of template and the number of amplification cycles were preliminarily optimized for each PCR reaction to avoid conditions of saturation.

mRNA and, for comparison, with probes for β-actin, ferritin, and r-protein (rp)-S6 mRNAs. All of these mRNAs revealed different translational modes. β-Actin mRNA appears to be almost completely associated with polysomes. This observation can be quantitatively expressed by the percentage of messenger on polysome (PMP), which is calculated by dividing the sum of the quantized signals of the first five fractions, corresponding to fast-sedimenting material containing polysomes, by the sum of the 10 fractions. In the case of β-actin the PMP value is 70. On the other hand, ferritin mRNA, known to be translationally repressed unless induced by iron uptake, is poorly associated with polysomes (PMP 18), and rp-S6 mRNA, whose association with polysomes is low unless stimulated by growth signals, is only partially associated with polysomes (PMP 40). In comparison with these mRNAs already characterized in other tissues, α-CaMKII mRNA appears to be mostly associated with polysomes (PMP 80), indicating its active translation in total brain. Because radioactive RT-PCR, rather than Northern blot analysis, is necessary to analyze the small amount of synaptic mRNA, we first set up the conditions for quantitative RT-PCR analysis on polysomal RNA extracted from total brain. To minimize experimental errors attributable to variations in the RT and PCR

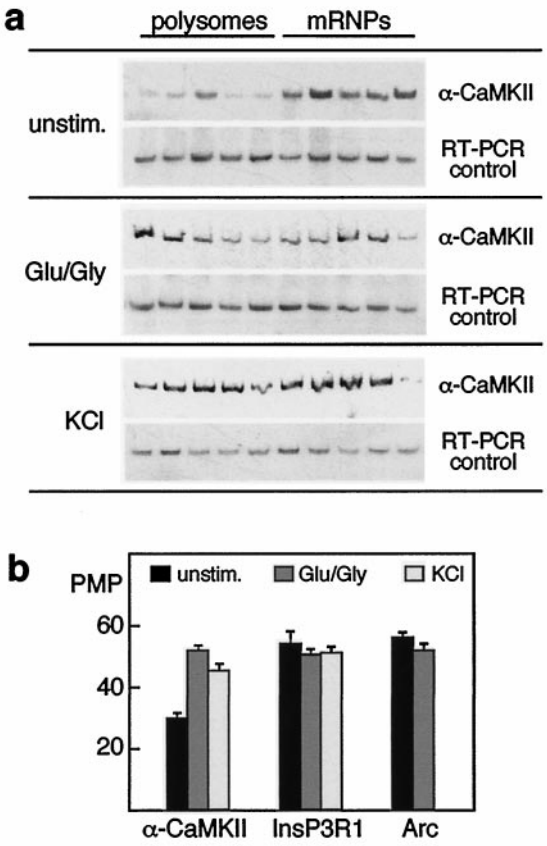

Figure 3. Polysome/mRNP distribution of α-CaMKII, InsP3R1, and Arc mRNAs in unstimulated and stimulated synaptosomes. *a*, Lysates from unstimulated and stimulated (*Glu/Gly, KCl*) synaptosomes were fractionated by sucrose gradient centrifugation. Ten fractions were collected from each gradient, each added with an equal amount of control RNA (see Materials and Methods) and then analyzed by quantitative radioactive RT-PCR for α-CaMKII mRNA and for control RNA. *b*, Five independent experiments were performed as in *a* and analyzed for α-CaMKII, InsP3R1, and Arc mRNAs. Radioactive signals in the polysomal and nonpolysomal fractions were quantified, corrected versus control RNA, and expressed as PMP. Values represent the mean ± SEM ($n = 5$ for α-CaMKII mRNA; $n = 3$ for InsP3R1 and Arc mRNAs).

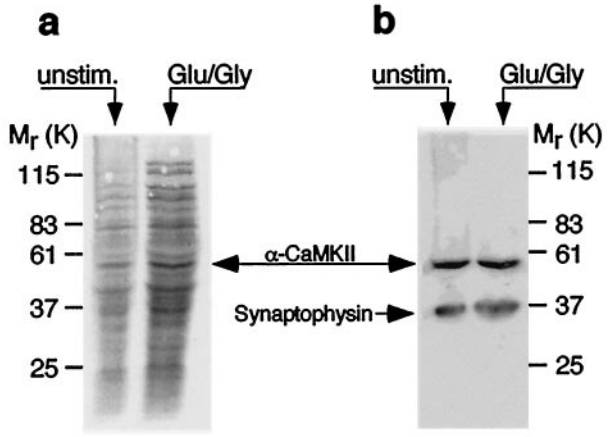

Figure 4. Protein synthesis in unstimulated and stimulated synaptosomes. *a*, Synaptosomes were labeled with [^{35}S]methionine/cysteine in the presence of chloramphenicol in unstimulated and Glu/Gly-stimulated conditions for 45 min. Equal amounts of proteins from the two samples were separated on an SDS-PAGE gel, transferred on a membrane, and autoradiographed (1 week exposure). The *arrow* points to a major band corresponding, for its molecular weight, to α-CaMKII protein. *b*, Western analysis of the same membrane with α-CaMKII-specific monoclonal antibody and, as control, with a synaptophysin antibody.

reactions, an equal amount of a synthetic RNA was added to each sample and used as an amplification control for the normalization of the obtained values before calculation of PMP. The translation profiles revealed by this method (Fig. 2*c*) are very similar to those obtained by Northern hybridization. This last result implies that mRNA translation can be studied in preparations containing low amounts of mRNA, as in the case of synaptosomes.

Radioactive RT-PCR analysis of gradient fractions from our synaptosomal preparation allowed us to evaluate the polysome association of synaptic α-CaMKII, InsP3R1, and Arc mRNAs. We found that, under basal (unstimulated) conditions, these three mRNAs are differentially associated with polysomes: PMP values of ~30, 50, and 52, respectively (Fig. 3; see below). The observation that α-CaMKII mRNA is rather inefficiently translated in synaptosomes (PMP 30 in synaptosomes vs 75 in total brain) prompted us to test whether its translation efficiency might be increased by chemical stimulation of the synapses. Preparations were analyzed under unstimulated conditions or after incubation in the presence of Glu/Gly or KCl. Figure 3*a* shows an example for α-CaMKII mRNA, and Figure 3*b* shows the quantized data obtained in at least three independent experiments. Interestingly, both kinds of stimulation resulted in an increased association of α-CaMKII mRNA with polysomes, Glu/Gly stimulation being more effective than KCl stimulation (PMP changes from 30 to 52 and to 40, respectively). In one experiment stimulation by glutamate only (glycine omitted) gave results similar to those of Glu/Gly stimulation (α-CaMKII PMP 48.5; data not shown), indicating that this translational regulation involves glutamate receptors. On the other hand, in the case of InsP3R1 and Arc mRNAs, the translational efficiency remained substantially unchanged under all tested conditions (Fig. 3*b*). Moreover, when synaptosomal polysomes were treated with EDTA, which is known to cause polysome dissociation, α-CaMKII, InsP3R1, and Arc mRNAs were indeed released from polysomes (data not shown).

The synaptosome preparations were also analyzed for ongoing protein synthesis in basal or stimulated conditions. The synaptosome fraction was equally divided, and the two samples were kept, respectively, in unstimulated and Gly/Gly-stimulated conditions. Both samples were incubated with [^{35}S]methionine/cysteine in the presence of chloramphenicol, which abolishes the mitochondrial protein synthesis (Polosa and Attardi, 1991; Rao and Steward, 1991). Equal amounts of total protein extracted from the two samples were analyzed by SDS-PAGE, transferred on a membrane, and exposed to x-ray film. The pattern observed, as shown in Figure 4*a*, is similar to the one previously described by Rao and Steward (1991). Moreover, comparison of unstimulated and stimulated synaptosomes shows a general increase of protein synthesis with stimulation. New proteins appear, some of them remain almost unchanged, whereas others show a substantial increase. The same experiment has been performed in the absence of chloramphenicol, in which a higher number of bands were observed, whereas no difference was observed using cycloheximide (data not shown). To verify that α-CaMKII protein was among the increased ones in the autoradiography shown in Figure 4*a*, a Western analysis was performed on the same membrane, using α-CaMKII antibody. As shown in Figure 4*b*, the increased

band of 54 kDa visualized with [^{35}S]methionine/cysteine incorporation corresponds to α-CaMKII. As expected, a similar increase is not observed in the Western blot, where we detect mostly the preexisting proteins that do not change during the 45 min of incubation.

DISCUSSION

In this work we studied the translation of α-CaMKII, InsP3R1, and Arc mRNAs in synaptosomes by analyzing their association with polysomes, a most suitable way for evaluating translational efficiency of individual mRNAs (Meyuhas et al., 1996). A similar approach has been previously used to analyze translation of fragile X mental retardation protein mRNA (Weiler and Greenough, 1993; Weiler et al., 1997). However, those results are somewhat impaired by limitations of the type of technique and of the crude synaptosome preparation used. In the present study we combined the use of a very sensitive molecular approach to measure the polysome and mRNP distribution of mRNA with the use of a highly purified synaptosome preparation to ensure that mRNA translation at the synapses reflects local protein synthesis and is not caused by contamination of cell body polyribosomes. The results obtained provide the first direct and clear evidence that, under basal (unstimulated) conditions, synaptic α-CaMKII, Arc, and InsP3R1 mRNAs are partly associated with polysomes and thus active in local translation at the synapses. Furthermore, in the case of α-CaMKII mRNA, we showed that its translational efficiency is increased by chemical stimulation of synaptosomes. This was seen as an increased association of α-CaMKII mRNA with polysomes and as an increase of new synthesized protein. These findings suggest that membrane signaling through glutamate receptors may promote an increase in α-CaMKII protein levels. It is significant that we observed this phenomenon for α-CaMKII, whose relevance in synaptic plasticity in the hippocampus has become well established through a variety of experimental approaches as NMDA receptor-dependent long-term potentiation in wild-type and α-CaMKII mutant mice (Silva et al., 1992; Thomas et al., 1994; Cho et al., 1998). The finding that the polysome association of InsP3R1 and Arc mRNAs does not change with synaptosome stimulation should not be taken as proof that their translation is not regulated. The synapse heterogeneity of our preparation may mask depolarization-induced translation regulation of some mRNAs that occurs only in specific types of synapses.

Much more work needs to be done to understand how local translation of particular mRNAs is regulated by synaptic stimulation and whether it contributes to synaptic plasticity. One mechanism possibly involved in the translational regulation of dendritic mRNAs might be the presence of specific repressor(s) that would mask the mRNAs, making them inaccessible to ribosomes, as it is known to occur for a number of mRNAs, for instance ferritin mRNA in mammals (Muckenthaler and Hentze, 1997) and oscar mRNA in *Drosophila* (Gunkel et al., 1998). After synaptic stimulation, the repressor(s) would be removed from the mRNAs, allowing their translation. So far no putative repressors of dendritic mRNA translation have been identified. Another possible mechanism for the control of translation might be the modulation of the poly(A) tail length at the 3′ end of the mRNAs (Preiss and Hentze, 1999). This possibility is in line with the finding that α-CaMKII mRNA contains in its 3′ untranslated region (UTR), in addition to a *cis*-acting signal for its localization in dendrites (Mayford et al., 1996), two *cis*-acting cytoplasmic polyadenylation elements (CPEs) implicated in the control of polyadenylation-induced translation of the mRNA by the CPE binding protein (Wu et al., 1998). These same authors have also shown that visual experience can cause a rapid polyadenylation of α-CaMKII mRNA and an increased amount of the encoded protein in the visual cortex of dark-reared rats.

The synaptosome preparation described here may be very useful to isolate putative regulatory factors binding the 5′ or 3′ UTR of synaptic mRNAs in unstimulated and stimulated conditions.

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