Metabotropic glutamate receptors trigger postsynaptic protein synthesis

(synaptic plasticity/polyribosomes/second messenger/protein kinase C/phosphatidylinositol)

IVAN JEANNE WEILER AND WILLIAM T. GREENOUGH

Departments of Psychology and Cell and Structural Biology, Neuroscience Program, and Beckman Institute, University of Illinois, Urbana-Champaign, IL 61801

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ABSTRACT K^+ depolarization or addition of glutamate to a synaptoneurosomes preparation triggers a rapid increase in size of polyribosomal aggregates isolated by centrifugation of lysate through 1 M sucrose. The profile of response to the glutamate analogues quisqualate, ibotenate, and 1-aminocyclopentane-1,3-dicarboxylate corresponds to that of metabotropic receptors. Glutamate stimulation is mimicked by the diacylglycerol analogue 1-oleoyl-2-acetyl-glycerol and by the protein kinase C activator phorbol dibutyrate. The phospholipase blockers 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate and quinacrine reduce the late phase of the response. The protein kinase C inhibitor calphostin C suppresses the response to 1-aminocyclopentane-1,3-dicarboxylate. These data indicate that glutamatergic synapses upregulate postsynaptic protein synthesis via metabotropic glutamate receptors coupled to the phosphatidylinositol second-messenger system. This mechanism could underlie the reported involvement of metabotropic glutamate receptors in long-term potentiation and other forms of neural plasticity.

The neurotransmitter glutamate exerts stimulatory effects on both ionotropic receptors, which activate ion channels, and metabotropic receptors, which exert their effects via second messengers (1–3). Several metabotropic receptors have been cloned (4, 5) and a role for these receptors in neuronal plasticity has been suggested, particularly in view of their predominance in early development (6–9); for example, the excitatory amino acid-stimulated phosphatidylinositol hydrolysis mediated by metabotropic glutamate receptors (mGluR) peaks in kitten striate cortex at the same time as sensitivity to visual deprivation (10). In a similar vein, at least some forms of long-term potentiation have recently been linked to mGluR (11–14).

The synaptoneurosomes suspension, shown by Verhage *et al.* (15) to retain metabolic integrity and the ability to release neurotransmitters for up to 4 hr makes it possible to sample the preparation repeatedly at intervals after stimulation. We reported (16) that K^+ depolarization triggers aggregation of postsynaptic polyribosomes and initiation of protein synthesis. By centrifugation of UV-crosslinked synaptoneurosomal lysates on continuous sucrose gradients, we showed that there was a relative increase in the number of less dense (newly loaded, protein-associated) polyribosomes following K^+ stimulation; further, there was transiently accelerated incorporation of [35 S]methionine into trichloroacetic acid-precipitable polypeptides. While Ca^{2+} chelators [40 μ M bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetate (BAPTA)] in the medium reduced the steady-state fraction of RNA bound up in polyribosomes, stimulation with 40 mM K^+ nevertheless resulted in a rapidly increased aggregation relative to the

lowered basal level, suggesting that release of intracellular sequestered Ca^{2+} stores mediated the effect. We now report that this polyribosomal aggregation occurs in response to mGluR agonists.

MATERIALS AND METHODS

Materials. BAPTA and calphostin C were obtained from Calbiochem. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 1-aminocyclopentane-1,3-dicarboxylate (ACPD) were from Tocris Neuramin. The 1*S*,3*R* and 1*R*,3*S* isomers of ACPD were a gift of Darryle D. Schoepp (Eli Lilly). All other reagents were from Sigma.

Synaptoneurosomes. Synaptoneurosomes were prepared as described (16). Briefly, the occipital–parietal cortex of groups of eight Long Evans rats (14–19 days old) was homogenized in buffer [125 mM NaCl/1 mM potassium acetate/100 mM sucrose/50 mM Hepes, pH 7.5/2 mM dithiothreitol with heparin (0.2 mg/ml) and cycloheximide (100 μ M)], filtered through nylon filters of decreasing pore size (final filter, 10- μ m pore), resuspended in 12 ml of buffer, and divided into aliquots. No additional calcium was added to the buffer. To each aliquot (at room temperature, with stirring) was added the appropriate combination of agonist with or without antagonist, and samples taken at intervals were lysed in 0.25% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent. Lysates were layered over 1 M sucrose in RK buffer [10 mM Tris, pH 7.6/1 mM potassium acetate/1.5 mM $MgCl_2$ /2 mM DTT/100 μ M sodium orthovanadate with heparin (0.5 mg/ml)], and centrifuged 11 min at 400,000 $\times g$ in a chilled Beckman TL-100 ultracentrifuge. The amount of RNA trapped in the polyribosomal pellet was compared to the total RNA (pellet plus supernatant, estimated on the basis of A_{260}). All animal handling and tissue preparation were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the Animal Welfare Act.

Statistics. All statistical comparisons involved SAS (SAS Institute, Cary, NC) general linear model one-factor (comparisons with $t = 0$ min) or two-factor (between-treatment comparisons) analysis with type III sum of squares used where appropriate, in cases with unequal n . In figures, comparisons are always between control and test responses within the same experiment; hence values in figures and Table 1 may differ. Each experiment used pooled tissues from eight animals but was treated statistically as a single experiment; n refers to number of experiments on which data are based. Individual group comparisons used the Student Newman–Keuls test, $P < 0.05$.

Abbreviations: ACPD, 1-aminocyclopentane-1,3-dicarboxylate; NMDA, *N*-methyl-D-aspartate; mGluR, metabotropic glutamate receptor(s); APV, 2-amino-5-phosphonovaleate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; NCDC, 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate.

RESULTS

When treated with either 40 mM K⁺ or 1 μ M glutamate, a portion of the RNA within synaptoneurosomes aggregated rapidly into polyribosomes, with a time course illustrated in Fig. 1. In untreated controls, polyribosomal RNA constituted roughly 20% of total RNA. There was a slight gradual increase in the polyribosomal fraction in the untreated suspension over the 20- to 30-min period of sampling; this was <10% of the $t = 0$ value (16). The addition of chelators such as 5 mM EGTA or 40 μ M BAPTA to the medium, although it lowered the basal level of polyribosomal loading, did not diminish the relative response to K⁺ depolarization (16). The response seemed to be biphasic; after the initial rapid increase at 1–2 min, there was often a pronounced decrease at 5 min and second peak at $\approx$ 10 min. The glutamate analogue NMDA, which activates Ca²⁺ channels, did not call forth the same response. We investigated a series of specific mGluR agonists; the data are summarized in Table 1.

Polyribosomal aggregation was effectively triggered by the selective mGluR agonist ACPD (17); the 1S,3R isomer was effective, but not the 1R,3S isomer; this difference has been shown to be diagnostic for the mGluR (18). Glutamate, quisqualate, and ibotenate were all effective at 10 μ M. NMDA was not effective at 10 μ M; at 1 mM it also slightly increased ribosomal aggregation. This pharmacological profile closely matches that described for mGluR (reviewed in ref. 9).

Antagonists of ionotropic receptors were next tested (Table 1). We found that the competitive NMDA receptor antagonist APV was ineffective in blocking the polyribosomal aggregation triggered by glutamate stimulation (Fig. 2), again suggesting that entry of external Ca²⁺ was not necessary.

The glutamate analogue quisqualate has been shown to be active at both ionotropic and metabotropic glutamate receptors, but only the ionotropic receptor is affected by the antagonist CNQX (19, 20). Since the response induced by 10 μ M quisqualate was not affected by 10 μ M CNQX, it is the

FIG. 1. Stimulation of synaptoneurosomes leads to a time-dependent increase in polyribosomal aggregation. Stimulation at $t = 0$ by either 40 mM K⁺ (●) ($n = 10$), 1 μ M glutamate (○) ($n = 10$), or 10 μ M *N*-methyl-D-aspartate (NMDA) (□) ($n = 6$). Ordinate, polyribosomal fraction of total RNA at $t = x$ expressed as a ratio to the same fraction at $t = 0$; abscissa, time; bars, SEM. For glutamate and K⁺, 1 min = 2 min = 10 min > 5 min > 20 min = 0 min (overall $P < 0.0001$ for time of sample); no interaction. For NMDA, no time points differ from $t = 0$.

Table 1. Effect of glutamate receptor agonists and antagonists on polyribosomal aggregation

Compound	Conc., μ M	<i>n</i>	Polyribosomal RNA fraction	
			2 min	10 min
Agonists				
Glutamate	10	7	1.15 $\pm$ 0.08	1.12 $\pm$ 0.06
	1	13	1.19 $\pm$ 0.03*	1.13 $\pm$ 0.05*
ACPD				
1 <i>S</i> ,3 <i>R</i>	10	14	1.25 $\pm$ 0.04*	1.15 $\pm$ 0.06*
1 <i>R</i> ,3 <i>S</i>	10	6	0.90 $\pm$ 0.07	0.90 $\pm$ 0.05
Quisqualate	10	12	1.28 $\pm$ 0.03*	1.29 $\pm$ 0.11*
Ibotenate	10	6	1.43 $\pm$ 0.09*	1.16 $\pm$ 0.09
NMDA	10	6	0.91 $\pm$ 0.04	1.02 $\pm$ 0.04
Antagonists (data pairs within same experiment)				
Quisqualate	10	3	1.24 $\pm$ 0.02 [†]	1.20 $\pm$ 0.09 [†]
+ CNQX	10	3	1.25 $\pm$ 0.02 [†]	1.11 $\pm$ 0.11 [†]
40 mM K ⁺	—	10	1.33 $\pm$ 0.04*	1.27 $\pm$ 0.05*
+ APV	50	5	1.35 $\pm$ 0.04*	1.35 $\pm$ 0.16*
+ quinacrine	100	6	1.18 $\pm$ 0.06	0.98 $\pm$ 0.05
+ NCDC	100	5	1.26 $\pm$ 0.09 [‡]	1.15 $\pm$ 0.06 [‡]
+ calphostin	2	2	1.30 $\pm$ 0.02	1.25 $\pm$ 0.07
Glutamate	1	4	1.21 $\pm$ 0.07*	1.10 $\pm$ 0.04
+ calphostin	2	4	1.09 $\pm$ 0.02	0.96 $\pm$ 0.04
Ibotenate	10	4	1.53 $\pm$ 0.08*	1.28 $\pm$ 0.04*
+ calphostin	2	4	1.26 $\pm$ 0.05*	1.03 $\pm$ 0.10
ACPD	10	3	1.35 $\pm$ 0.06	1.15 $\pm$ 0.12
+ calphostin	2	3	1.08 $\pm$ 0.03	1.06 $\pm$ 0.07

Polyribosomal RNA as a fraction of total RNA at $t = x$, expressed as a ratio to the same fraction at $t = 0$. Values are means $\pm$ SEM. APV, 2-amino-5-phosphonopentanoate; NCDC, 2-nitro-4-carboxyphenyl-*N,N*-diphenylcarbamate.

*Differs from $t = 0$; $P < 0.05$.

†Significant effect of treatment, but no difference between treatments in 2 $\times$ 3 analysis of variance.

‡In comparison with simultaneous K⁺ group, significant effect of treatment, significant reduction of K⁺ effect by NCDC; SAS general linear model Student Newman-Keuls comparisons.

metabotropic quisqualate receptor which appears to be responsible for the polyribosomal aggregation.

Since mGluR (particularly mGluR1 α) have been linked to accumulation of inositol phosphates in the rat neocortex (5, 8, 21, 22), we used a number of pharmacological agents to probe the phosphatidylinositol hydrolysis system. We first

FIG. 2. Stimulation of synaptoneurosomes by 1 μ M glutamate (—●—) was not blocked by simultaneous administration of 100 μ M APV (---●---). In this and all subsequent figures, the standard comparison curve for glutamate is taken from the same experiments in which the specific agent was tested.

FIG. 3. Quinacrine (100 μ M), a phospholipase blocker (24), decreased the polyribosomal aggregation response to 40 mM K⁺, particularly in its later phase. ●, 40 mM K⁺; ○, quinacrine plus 40 mM K⁺. Quinacrine $\neq$ K⁺, $P < 0.0005$; $n = 6$.

investigated the effects of two phospholipase blockers on the time course of polyribosomal aggregation. NCDC (23) and quinacrine (24) showed a very slight effect on the initial (1–2 min) peak but more pronounced suppression at 5 min and later (Table 1 and Fig. 3; for K⁺ vs. NCDC, $F_{1,32} = 6.00$, $P < 0.05$; for K⁺ vs. quinacrine, $F_{1,53} = 14.55$, $P < 0.01$). The difference between early and late effects, which we observed with both blockers, suggests (as does the biphasic nature of the response) that more than one process may be involved.

To mimic the effects of phosphatidylinositol hydrolysis without the involvement of glutamate itself, we administered 1-oleoyl-2-acetyl-glycerol, a cell-permeant analogue of diacylglycerol (25) which stimulates protein kinase C. The strong aggregation response of polyribosomes and mRNA to this stimulation, parallel to the biphasic response to glutamate in the same experiments (Fig. 4), suggests strongly that protein kinase C, which has been implicated in plastic synaptic change (26), is probably one of the enzymes involved in

FIG. 4. 1-Oleoyl-2-acetyl-glycerol (OAG, 5 μ M), a cell-permeant diacylglycerol analogue (25), was suspended in 1% dimethyl sulfoxide, and on the day of the experiment was diluted and sonicated for 10 min before addition to the synaptoneurosomes suspension (final dilution, 5 μ g/ml). ○, OAG-treated synaptoneurosomes; ●, parallel samples of synaptoneurosomes with 1 μ M glutamate. Glu = OAG; Glu and OAG 2 min > 0 min, $P < 0.0001$, $n = 5$.

FIG. 5. Synaptoneurosomes were stimulated with 1 μ M glutamate (●) or 1 μ M phorbol 12,13-dibutyrate (○); $n = 6$.

signaling this process. We also tested 4 β -phorbol 12,13-dibutyrate, an activator of protein kinase C at the diacylglycerol binding site. Polyribosomal aggregation increased rapidly in response to 1 μ M 4 β -phorbol 12,13-dibutyrate, with a markedly biphasic time course, peaking at 1 min and 5 min (Fig. 5).

To block protein kinase C, we employed calphostin C, a highly specific inhibitor which interacts with the regulatory domain (27). The greatest effect, >80% inhibition by 2 μ M calphostin, was seen against the selective metabotropic receptor agonist ACPD (Fig. 6; $F_{1,30} = 11.50$, $P < 0.01$). The response to 10 μ M ibotenate and glutamate was reduced by about half (Table 1; for ibotenate, $F_{1,18} = 13.21$, $P < 0.01$; for glutamate, $F_{1,18} = 8.25$, $P < 0.05$). Thus, when only the mGluR were stimulated, as was the case with ACPD, a protein kinase blocker almost completely suppressed the polyribosomal aggregation. Within the same experiments, however, calphostin did not block the polyribosomal aggregation triggered by 40 mM K⁺, suggesting that more than one activation system may be triggered by K⁺ depolarization.

DISCUSSION

Postsynaptic polyribosomal aggregates have been postulated to be associated with synaptic plasticity (28, 29), and protein synthesis has been implicated in many plastic neural processes (30–33). If protein synthesis is involved in synaptic plasticity, then the need for initiation of plastic change at specific synaptic nodes in the neural network demands lo-

FIG. 6. Calphostin C (2 μ M), a protein kinase C blocker, was added to half the synaptoneurosomes; the response to 10 μ M ACPD (○) was compared with that of the suspension not treated with calphostin (●). ACPD > ACPD + calphostin, $P < 0.005$; $n = 6, 3$.

calized control. This could be provided by activity-dependent protein translation on postsynaptic polyribosomal aggregates.

To observe the rapid change in polyribosomal loading, we have taken serial samples from a suspension of synaptoneurosomes. It should be emphasized that synaptoneurosomes preparations, which have proven useful in a multitude of investigations of neurotransmitter effects, are always a mixture of different subcellular particles (excluding cell nuclei). Thus glial or other small neuronal fragments are always present and might contribute to the phenomenon we describe. The facts that they are responsive to depolarization by K^+ and respond specifically to metabotropic glutamate analogues, however, suggest that synaptosomes are, at the very least, the major responsive component in our system. There is considerable variation between experiments, which we have not been able to eliminate. For this reason we have compared effects produced by agonists and antagonists with standard positive responses only within the same experiments. Thus, values for the standard response to glutamate, for example, in a given group of three to five experiments, may differ from the mean of all experiments pooled for Table 1.

We have previously shown that depolarization of synaptoneurosomes leads to the loading of ribosomes onto mRNA so as to form polyribosomal aggregates (16). Incorporation of [^{35}S]methionine was also transiently accelerated, suggesting that protein translation was being triggered. We have now shown that this activation is effected by a neurotransmitter, glutamate, acting on metabotropic receptors, not on ion channel-coupled receptors; the sensitivity of polyribosomal loading to various glutamate analogues corresponds to that typical of metabotropic receptors (9). Interference with phospholipid hydrolysis, by phospholipase blockers, markedly reduced the polyribosomal response. The later phase of the response was more sensitive to this inhibition, suggesting that multiple processes were operating.

Our data suggest that phosphatidylinositol hydrolysis, by activation of protein kinase C via diacylglycerol production, might lead to rapid phosphorylation of some factors controlling protein synthesis, since both a diacylglycerol analogue, 1-oleoyl-2-acetyl-glycerol, and a protein kinase C activator, 4 β -phorbol 12,13-dibutyrate, effectively mimic activation by glutamate. This is not to ignore the role played by increased intracellular Ca^{2+} (34, 35), as it participates in activation of protein kinase C and calmodulin-regulated kinases (36). In the case of activation by the exclusively metabotropic agonist ACPD, we were able to suppress the response entirely with calphostin C, a protein kinase C blocker. This did not greatly reduce the response to K^+ depolarization, however, suggesting that more than one process was in operation. We propose that mGluR-induced hydrolysis of membrane phospholipids activates a second-messenger pathway which interacts with ion channel-generated events in regulating local translation of proteins near synapses.

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