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Short communication

Ageing is associated with changes in glutamate release, protein tyrosine kinase and Ca^{2+}/calmodulin-dependent protein kinase II in rat hippocampus

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Abstract

We have used synaptosomes prepared from rat hippocampus to investigate the role of protein tyrosine kinase and Ca^{2+}/calmodulin-dependent protein kinase II in modulating glutamate release in young animals and to investigate possible parallel age-related changes in release and kinase activity. We report that depolarization of synaptosomes with 40 mM KCl, which stimulated glutamate release, also significantly increased activity of both kinases, while the protein tyrosine kinase inhibitor, genistein and the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, KN62 (1-(*N*,*O*-bis[5-isoquinolinesulfonyl]-*N*-methyl-tyrosyl)-4-phenylpiperaxine) decreased K^+-stimulated, Ca^{2+}-dependent release of glutamate. K^+-stimulated release of glutamate was significantly decreased in hippocampal synaptosomes prepared from aged, compared to young, animals. In parallel with these changes in release, we report an age-related decrease in activities of both protein tyrosine kinase and Ca^{2+}/calmodulin-dependent protein kinase II. We conclude that these kinases play a role in modulating release of glutamate in hippocampus and that the age-related decrease in glutamate release may be partly due to an age-related decrease in kinase activities.

Keywords: Glutamate release; Protein tyrosine kinase; Ca^{2+}/calmodulin-dependent protein kinase II; Phosphorylation; Synaptophysin; Synapsin; Hippocampus; Ageing

1. Introduction

Glutamate, the transmitter in several pathways in the hippocampus, is the major excitatory transmitter in the brain. While it has been long acknowledged that increased intracellular Ca^{2+} concentration plays a pivotal role in the release of transmitters, including glutamate, the detailed sequence of events following Ca^{2+} influx remains unclear. Phosphorylation of, at least, some synaptic vesicle proteins plays a role in this sequence. Thus it has been shown that phosphorylation of synapsin by Ca^{2+}/calmodulin-dependent protein kinase II results in liberation of the synaptic vesicles from the actin meshwork, which then become available for docking and fusion (Ceccaldi et al., 1995). Similarly, phosphorylation of synaptophysin on serine residues has been shown to accompany depolarization (Rubenstein et al., 1993), while depolarization of synaptosomes prepared from rat forebrain is associated with increased activity of protein tyrosine kinase (Woodrow et al., 1992).

Release of several neurotransmitters decreases with increasing age but in the case of excitatory amino acid transmitters, findings are inconclusive and even contradictory with the age-related change apparently being determined by the brain area and species studied (e.g. Meldrum et al., 1992; Aprikyan and Gekchyan, 1988; Saransaari and Oja, 1994). We have shown that there is an age-related decrease in glutamate release in slices prepared from dentate gyrus (Lynch and Voss, 1994) and in synaptosomes prepared from whole hippocampus (McGahon and Lynch, unpublished). The underlying cause of the age-related change is not known. In this study we investigated, in young and aged rats, activities of Ca^{2+}/calmodulin-dependent kinase II and protein tyrosine kinase, two kinases which play a role in release and whose activities are decreased in at least one age-related condition, Alzheimer's disease (Murray et al., 1994). The aims were two-fold: (a) to investigate the role of protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II in modulating glutamate release in synaptosomes prepared from rat hippocam-

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pus; and (b) to investigate, in parallel, age-related changes in kinase activities and glutamate release.

2. Materials and methods

Male Wistar rats (aged 4 or 22 months) were killed by cervical dislocation. The hippocampus was dissected free, tissue was homogenized in 0.32 M ice-cold sucrose and the crude synaptosomal pellet, P_2, was prepared (Lynch and Voss, 1994). Synaptosomes were resuspended in Krebs solution (composition in mM: NaCl, 136; KCl 2.54; KH_2PO_4, 1.18; $MgSO_4 \cdot 7H_2O$, 1.18; $NaHCO_3$, 16; glucose 10) containing 2 mM $CaCl_2$ and incubated for 15 min at 37°C in the presence of [^{3}H]glutamate (Amersham, UK: specific activity 0.74–2.2 TBq/mmol; final concentration 5×10^{-7} mM). Release was assessed as previously described (Lynch and Voss, 1994). Briefly, tissue was aliquotted onto Millipore filters (0.45 μm), rinsed under vacuum and then incubated for 5 min at 37°C in 250 μl oxygenated Krebs solution with or without added $CaCl_2$ (2 mM). The incubation step was repeated in the presence of 40 mM K^+ to depolarize the synaptosomes. Filtrate was collected for scintillation counting to estimate spontaneous and stimulated glutamate release, respectively. In some cases the protein tyrosine kinase inhibitor, genistein (final concentration 50 μM; Sigma, UK) or the Ca^{2+}/calmodulin-dependent kinase II inhibitor, KN62 ([1-[*N*,*O*-bis(1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine]); final concentration 5 μM; Sigma) was added during the second incubation. We found that, under depolarized conditions, at least 80% of the radiolabel was recovered as [^{3}H]glutamate.

Protein tyrosine kinase activity was also examined in P_2. Aliquots of resuspended synaptosomes were added to incubation medium (composition in mM: Tris-HCl, 20, pH 7.4; $MgCl_2$, 20; $MnCl_2$, 2; EDTA, 0.5; EGTA, 1; ouabain, 1; sodium vanadate, 1; dithiothreitol, 0.1), containing adenosine-5-triphosphate ([^{32}P]ATP; 110 TBq/mmol; Amersham, UK). The final solution contained 0.1% Triton X-100 (total volume 100 μl) and all estimations were made in both the presence and absence of the specific protein tyrosine kinase substrate, poly(Glu^{80},Tyr^{20}), and in the presence and absence of K^+ (40 mM). The reaction was started by addition of synaptosomes, incubation continued for 10 min at 37°C and the reaction was stopped by addition of 10 mM ATP/0.25 mM EDTA. An aliquot (55 μl) of the reaction material was immediately spotted onto Whatman 3MM filter paper and washed 5 times in trichloroacetic acid (10% w/v)/sodium pyrophosphate and once in 95% ethanol. Dried filter papers were added to scintillant and radioactivity counted. Results were expressed as nmol [^{32}P] incorporated/μg protein. Protein concentration was assessed according to the method of Bradford (1976).

For analysis of Ca^{2+}/calmodulin-dependent kinase II activity, synaptosomes were resuspended in 20 mM Tris buffer (pH 7.4) containing 10 mM EDTA, 2 mM EGTA, 100 mM 1β-glycerophosphate, 50 μg/ml phenylmethylsulphonylfluoride, 0.1 U/ml aprotonin, 2 μM pepstatin, 1 mg/ml leupeptin, 0.1 mg/ml ovalbumin and 0.25 M sucrose. The reaction was started by addition of 10 μl synaptosomal suspension to incubation medium (concentration in mM: EGTA, 0.5; $MgCl_2$, 12.5; HEPES, 20; pH 7.5) containing 100 nM [^{32}P]ATP, and all estimations were made in the presence and absence of the synthetic peptide substrate for Ca^{2+}/calmodulin-dependent kinase

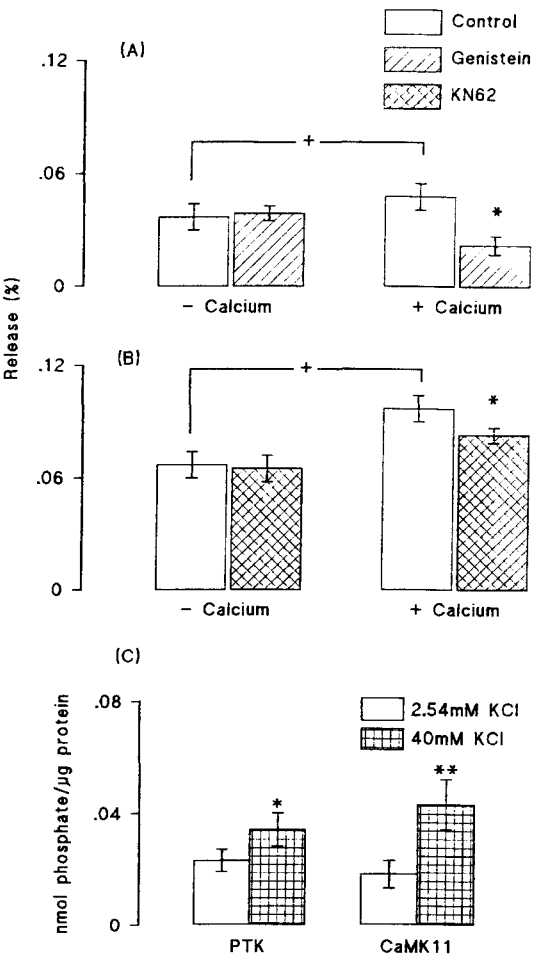

Fig. 1. (A,B) K^+-stimulated release of [^{3}H]glutamate (i.e. release in the presence of K^+ minus spontaneous release) in synaptosomes prepared from young rats was increased by addition of $CaCl_2$ ($^+P < 0.05$, compared to release in the absence of $CaCl_2$; Student's *t*-test for independent means). Both genistein (A; 50 μM) and KN62 (B; 5 μM) significantly inhibited K^+-stimulated release in the presence of $CaCl_2$ ($^*P < 0.05$; Student's *t*-test for paired means; $n = 9$ in KN62 experiments and $n = 11$ in genistein experiments). Mean spontaneous release (±S.E.M.) for A and B was 0.07 (±0.004) and 0.051 (±0.004), respectively. Data (means±S.E.M.) for K^+-stimulated release are given as a percentage of the total amount of radiolabel present at the start of that incubation period. (C) Addition of K^+ (40 mM) to the incubation medium significantly increased activity of both protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II ($^*P < 0.05$; $^{**}P < 0.01$; Student's *t*-test for paired means; $n = 11$).

II, autocamtide-2, and in the presence or absence of K^+ (40 mM). The final volume was 50 μl. Incubation continued at 30°C for 5 min and the reaction was terminated by application of 25 μl of the incubation material to Whatman 3MM filter papers, which were immediately added to 10 ml trichloroacetic acid (10%) and subsequently washed twice in fresh trichloroacetic acid (10%) for 10 min. Dried filter papers were added to scintillant and radioactivity counted.

Student's *t*-test was used to evaluate the data. Either the test for dependent means or independent means was used as appropriate. The α level was chosen as 0.05.

3. Results

Spontaneous and K^+-stimulated release of [^{3}H]glutamate in synaptosomes prepared from whole hippocampus was examined in the absence and presence of Ca^{2+}. Spontaneous release was similar in both conditions (data not shown). Addition of 40 mM K^+ led to an approximate two-fold increase in release (above spontaneous) in the absence of Ca^{2+} and a three-fold increase in its presence.

Fig. 1A,B illustrates that K^+-stimulated release (i.e. release in the presence of 40 mM KCl minus spontaneous release) was significantly greater in the presence of Ca^{2+} compared to in its absence ($P < 0.05$). While genistein (50 μM) had no significant effect on K^+-stimulated [^{3}H]glutamate release from hippocampal synaptosomes in the absence of Ca^{2+}, it significantly decreased release in its presence (Fig. 1A; $P < 0.05$). Similarly, KN62 (5 μM) significantly decreased K^+-stimulated glutamate release in the presence of Ca^{2+} (Fig. 1B; $P < 0.05$) but had no effect in its absence.

In parallel with the K^+-induced increase in release of glutamate, we found that K^+ significantly increased activity of protein tyrosine kinase (Fig. 1C; $P < 0.05$). Similarly, addition of 40 mM K^+ to the incubation medium significantly increased activity of Ca^{2+}/calmodulin-dependent kinase II ($P < 0.01$). These data indicate that activation of both kinases is, like transmitter release, depolarization-dependent.

Figs. 1A,B and 2A indicate that addition of 40 mM K^+ to Ca^{2+}-containing incubation medium resulted in a significant increase in [^{3}H]glutamate release from hippocampal synaptosomes prepared from 4-month-old animals. In marked contrast to this effect, there was no increase in [^{3}H]glutamate release in the presence of Ca^{2+} above that observed in its absence in synaptosomes prepared from 22-month-old animals (Fig. 2A). In parallel with this compromised release in aged animals, there was an age-related decrease in K^+-stimulated activity of both protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II ($P < 0.05$ in the case of protein tyrosine kinase and $P < 0.01$ in the case of Ca^{2+}/calmodulin-dependent kinase II; Fig. 2B).

4. Discussion

The data described here indicate that (a) depolarization with 40 mM KCl increased both glutamate release and activity of Ca^{2+}/calmodulin-dependent kinase II and protein tyrosine kinase, while inhibitors of protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II inhibited glutamate release in hippocampal synaptosomes, and that (b) activity of both kinases decreased with age in parallel with a decrease in K^+-stimulated, Ca^{2+}-dependent release.

The observation that glutamate release was decreased by the Ca^{2+}/calmodulin-dependent kinase II inhibitor, KN62, indicates that K^+-stimulated release in hippocampal

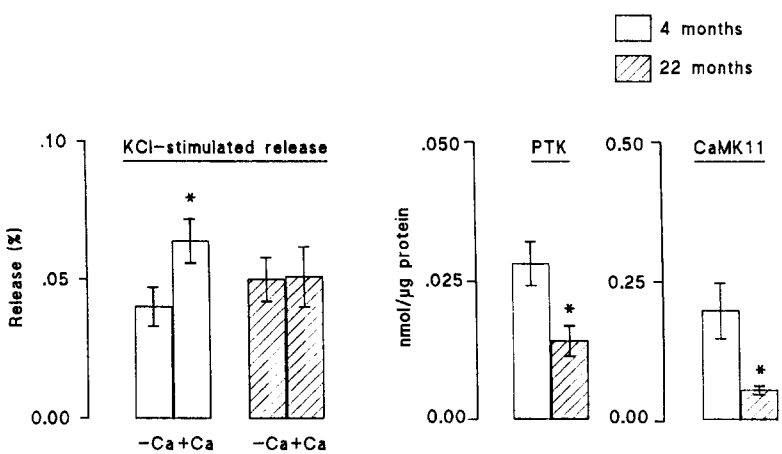

Fig. 2. K^+-stimulated release of [^{3}H]glutamate in synaptosomes prepared from young rats was increased by addition of $CaCl_2$ (* $P < 0.01$, compared to release in the absence of $CaCl_2$; Student's *t*-test for independent means; $n = 8$); this effect was attenuated in synaptosomes prepared from aged rats. Mean spontaneous release (±S.E.M.) was 0.058 (±0.007) and 0.052 (±0.004) in young and aged rats, respectively. Activity of both kinases was significantly reduced in synaptosomes prepared from aged, compared to young, animals (* $P < 0.05$; Student's *t*-test for independent means; $n = 6$).

synaptosomes involves activation of Ca^{2+}/calmodulin-dependent kinase II. It has previously been shown that injection of Ca^{2+}/calmodulin-dependent kinase II into synaptosomes increased release of transmitter, while peptidergic inhibitors of Ca^{2+}/calmodulin-dependent kinase II blocked transmitter release (Nichols et al., 1990), in situ phosphorylation of synapsin and excitatory synaptic responses in area CA1 of the hippocampus (Waxham et al., 1993). Although the substrate involved in modulation of release was not identified in the present experiments, it is likely that this is synapsin, the major presynaptic substrate for Ca^{2+}/calmodulin-dependent kinase II. Synapsin modulates release depending on its phosphorylation state. Thus increased phosphorylation of synapsin accompanied increased transmitter release (Nichols et al., 1990) while introduction of the dephosphorylated form of synapsin into synaptosomes decreased K^+-stimulated glutamate release. Although synapsin is the major substrate for Ca^{2+}/calmodulin-dependent kinase II, it has been shown recently that synaptophysin is also a substrate (Rubenstein et al., 1993). Serine phosphorylation of synaptophysin was shown to increase following K^+-induced depolarization; this was enhanced by the presence of Ca^{2+}, calmodulin and Ca^{2+}/calmodulin-dependent kinase II in the medium, and inhibited by Ca^{2+}/calmodulin-dependent kinase II inhibitory peptides. Therefore phosphorylation of synaptophysin as well as synapsin may be compromised by Ca^{2+}/calmodulin-dependent kinase II inhibitors. We must conclude that reduced phosphorylation of both synaptic vesicle proteins may contribute to the decrease in glutamate release induced by KN62 which we describe here.

The findings of the present experiments indicate that inhibiting activity of protein tyrosine kinase by genistein exerts an inhibitory effect on glutamate release in synaptosomes prepared from hippocampus. The major presynaptic substrate for protein tyrosine kinase is synaptophysin (Pang et al., 1988), but the functional significance of tyrosine phosphorylation of synaptophysin remains unclear. A recent report indicated that synaptophysin was phosphorylated on tyrosine residues following depolarization (albeit to a very small degree; Rubenstein et al., 1993), suggesting that transmitter release may be associated with tyrosine phosphorylation of synaptophysin. The contribution of synaptophysin to release probably lies in its role in vesicle fusion (Thomas and Betz, 1990) but whether or not phosphorylation of the protein is required for this process is unknown. The observation that genistein exerts an inhibitory effect on release indicates that phosphorylation of a protein tyrosine kinase substrate, perhaps synaptophysin, modulates glutamate release in hippocampus.

The present demonstration that K^+-stimulated release of glutamate was compromised in hippocampal synaptosomes prepared from aged, compared to young, animals supports our earlier observation indicating an age-related decrease in release in slices prepared from dentate gyrus (Lynch and Voss, 1994). These results are in agreement with previous reports investigating changes in release in preparations obtained from aged rats (Aprikyan and Gekchyan, 1988; Taylor and Griffith, 1993), but not aged mice (Saransaari and Oja, 1994). However, one report indicates that there was an age-related *increase* in [^{3}H]D-aspartate release in slices prepared from rat hippocampus (Meldrum et al., 1992). There are a number of factors which might contribute to the differences between this observation and the present findings. First, in the present study release of [^{3}H]glutamate was assessed by stimulating synaptosomes with 40 mM KCl, while in the study of Meldrum and colleagues, release of [^{3}H]D-aspartate was assessed by electrically stimulating hippocampal slices. While it would be surprising to find that the mode of stimulation affected the response, it is of interest that an increase in [^{3}H]D-aspartate release was observed only at one of a range of stimulus intensities. It is likely that the use of different radiolabels contributes significantly to the difference in findings since we have previously found that both uptake and release of [^{3}H]glutamate and [^{3}H]D-aspartate from hippocampal preparations were not the same (Feasey et al., 1986). The variation in results may also reflect the different strains of rat used (Fischer 344 vs. Wistar rats) since strain differences in hippocampal function, particularly in aged rats have been reported (Diana et al., 1994). The fact that analysis was performed in slices prepared from 28–30-month-old rats in one study (Meldrum et al., 1992) compared to synaptosomes prepared from 22-month-old rats in the present study, may also contribute to the differences observed.

Depolarization with K^+ in the presence of Ca^{2+} led to an increase in release of about 60% above release in its absence, in synaptosomes prepared from young animals, compared to an equivalent increase of less than 10% in aged animals. The basis of this decrease has not been established but it is paralleled by a decrease in activity of Ca^{2+} currents in hippocampus (Reynolds and Carlen, 1989) and a decrease in synaptic density, at least in dentate gyrus (Saito et al., 1994). On the basis of the present experiments, indicating a concomitant age-related decrease in K^+-stimulated glutamate release and K^+-stimulated activity of both protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II, we suggest that the compromised activity of the kinases may also contribute to the age-related decrease in glutamate release.

Our data indicate a role for protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II in K^+-stimulated release of glutamate from hippocampal synaptosomes; indirect evidence suggests that among the substrates for these kinases are the synaptic vesicle proteins, synaptophysin and synapsin. We demonstrate that release of glutamate from hippocampal synaptosomes is compromised in aged, compared to young animals, and we propose that this may be derived, in part, from an age-related decrease in K^+-stimulated activity of protein tyrosine kinase and Ca^{2+}/calmodulin-dependent kinase II in synaptosomes.

Acknowledgements

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