RESEARCH COMMUNICATION

The secreted form of the Alzheimer's β -amyloid precursor protein stimulates a membrane-associated guanylate cyclase

Steven W. BARGER* and Mark P. MATTSON†‡

*Sanders-Brown Center on Aging and †Department of Anatomy and Neurobiology, University of Kentucky, Lexington, KY 40536-0230, U.S.A.

We previously demonstrated that secreted forms of the Alzheimer's β -amyloid precursor protein (sAPP) elevate cyclic GMP (cGMP) in primary neuronal cultures and that this effect is responsible for the modulation of neuronal calcium homeostasis by sAPP. We have investigated further the mechanism by which sAPP elevates cGMP. Inhibition of the formation of nitric oxide or carbon monoxide did not affect the ability of sAPP to lower rapidly intraneuronal calcium levels or elevate

cGMP, suggesting that sAPP does not activate a soluble (cytosolic) guanylate cyclase. A dose-dependent stimulation of cGMP formation by sAPP was observed in brain membrane preparations. The stimulation was also dependent on the presence of ATP. These data suggest that sAPP activates a membrane-associated guanylate cyclase, perhaps similar to those present in the receptors for the natriuretic peptides and sperm motility factors.

INTRODUCTION

The β -amyloid precursor protein (β APP) is perhaps best known as the source of the β -peptide that accumulates as insoluble plaques in age-associated dementias. However, β APP has been ascribed various functions in the normal brain (reviewed in [1,2]). Proteolytic processing alternative to that which generates β -amyloid peptide can liberate from the cell surface most of the extracellular portion of the protein. This soluble β APP (sAPP) appears to exert meaningful effects on neighbouring cells, including enhancement of mitogenesis [3], neurite outgrowth [4], survival [5,6] and synapse formation or stability [7]. At least some of these effects appear to result from the activation of cyclic GMP (cGMP)-dependent protein kinase [8]. β APP is axonally localized [9,10], and the release of sAPP is responsive to neuronal activity and to the activation of specific neurotransmitter receptors [11,12]. These data suggest that sAPP may play a neuromodulatory role at the synapse and urge elucidation of the mechanisms involved.

Two types of guanylate cyclase are responsible for agonist-stimulated formation of cGMP. The most ubiquitous form is a haem-containing, soluble cyclase that is activated by the noxious gases NO and CO. This cyclase is responsible for generation of cGMP in response to elevation of intracellular calcium levels ($[Ca^{2+}]_i$), a stimulus that activates the calmodulin-dependent enzyme NO synthase. A second class of guanylate cyclase includes those present in the cytoplasmic portion of the receptors for various peptide hormones [13]. The situation of these cyclases as integral membrane proteins accounts for their segregation from the soluble cyclases during cell fractionation preparations. We used such a cell fractionation approach, as well as pharmacological manipulations, to distinguish between soluble and membrane-associated guanylate cyclases in mediation of the actions of sAPP.

EXPERIMENTAL

Fura 2 microfluorescent imaging was used to measure $[Ca^{2+}]_i$ in the somata of hippocampal neurons cultured from 18-day-old

embryonic rats as described previously [8]; each experimental condition depicted represents the mean $[Ca^{2+}]_i$ of 17–47 neurons from at least two platings. The sAPP derived from β APP695 was purified as described in [8] and was added to the cultures during image acquisition in the presence or absence of N^G -nitro-L-arginine (N-Arg) (Sigma), N^G -methyl-L-arginine (Sigma) or Zn-protoporphyrin IX (ZnPPIX) (Aldrich).

cGMP assays of whole cells were performed as described previously [8]. For measurement of particulate cyclase, membranes were prepared from postnatal (≤ 24 -h-old) Sprague-Dawley rat hippocampus or cortex. Tissue was homogenized in a sucrose-containing buffer [250 mM sucrose/50 mM Tris/HCl/1 mM EDTA/1 mM β -mercaptoethanol (pH 8.0)] on ice with a Dounce Teflon pestle. After a low-speed centrifugation to remove nuclei and other large contaminants, the homogenate was subjected to centrifugation at 100 000 g for 60 min at 4 °C. The pellet was washed briefly with a solution of 50 mM Tris/HCl (pH 7.0) and 1 mM β -mercaptoethanol, then resuspended in the same. Guanylate cyclase activity was measured by incubation of membrane with GTP, followed by radioimmunoassay of cGMP as described in [8]. Standard assays involved warming of the membrane to 37 °C in 50 μ l of a 2 $\times$ concentrated mixture of all buffer components excepting GTP and sAPP: 40 mM Tris/HCl/8 mM $MgCl_2$ /2 mM isobutylmethylxanthine/11 μ g/ml phosphocreatine/1 μ g/ml creatine kinase. The reaction was initiated by the simultaneous addition of 25 μ l of 4 mM GTP and 25 μ l of sAPP or control saline. Unless otherwise indicated, the pH was 7.0, and ATP was included to a final concentration of 1 mM. The reaction was stopped by the addition of ice-cold 50 mM sodium acetate, pH 4.0. Samples were stored frozen and cleared by centrifugation before radioimmunoassay of cGMP.

RESULTS

The addition of sAPP to cultured hippocampal neurons is marked by a rapid decrease in $[Ca^{2+}]_i$ [8,14]. We have previously determined that this response is correlated with an elevation of

Abbreviations used: β APP, β -amyloid precursor protein; $[Ca^{2+}]_i$, free intracellular calcium concentration; cGMP, cyclic GMP; N-Arg, N^G -nitro-L-arginine; sAPP, secreted form of β APP; ZnPPIX, Zn-protoporphyrin IX.

‡ To whom correspondence should be addressed.

Figure 1 Depression of $[Ca^{2+}]_i$ by sAPP is independent of NO and CO

Rat hippocampal neurons were loaded with the calcium-indicator dye fura 2 and subjected to microfluorometric image analysis during the application of 50 pM sAPP (arrows). Responses of control cells are compared with those of cells pretreated with 100 μ M N-Arg (a) or 10 μ M ZnPPiX (b). Each trace represents the mean for 19–32 cells from three cultures. Neither drug treatment resulted in a $[Ca^{2+}]_i$ depression that was significantly different from the response to sAPP alone ($P > 0.1$, N-Arg versus control (Con); $P > 0.4$, ZnPPiX versus control).

cGMP levels and is dependent upon cGMP-dependent kinase [8]. Therefore we used the depression of $[Ca^{2+}]_i$ as a sensitive index of sAPP activity. To test the involvement of soluble guanylate cyclase in this activity, we monitored sAPP-stimulated depression of $[Ca^{2+}]_i$ under conditions expected to block formation of noxious gases. Soluble guanylate cyclase can be activated by NO, which is liberated enzymically from L-Arg by a calmodulin-dependent enzyme. Several analogues of Arg have been determined to inhibit this reaction competitively in neurons [15]. We have previously determined that 100 μ M N-Arg can alter the effects of glutamate on $[Ca^{2+}]_i$ [8] and cGMP in hippocampal neurons. For instance, 30 μ M glutamate caused an approx. 4.5-fold elevation of cGMP in these cultures, and this effect was completely blocked by 100 μ M N-Arg. However, pretreatment with 100 μ M N-Arg did not affect the depression of $[Ca^{2+}]_i$ stimulated by sAPP (Figure 1a). Similar results were obtained with *N*^G-methyl-L-Arg (results not shown). In some systems, rapid responses of soluble guanylate cyclase to signals initiated by extracellular agonists are dependent on CO rather than on NO [16,17]. The generation of CO under these conditions seems to involve the activity of haem oxygenase. Accordingly, we assayed the activity of sAPP in the presence of a haem oxygenase

Figure 2 Stimulation of membrane-associated guanylate cyclase activity of sAPP

(a) Dependence on pH of the sAPP-stimulation of membrane guanylate cyclase activity. Membranes were prepared from cerebral cortex and assayed for the formation of cGMP with or without the addition of 10 nM sAPP. Reactions utilizing 40 μ g of membrane protein were performed for 4 min at the indicated pH. Values represent the mean of triplicate determinations. (b) Dose response of the sAPP-stimulation of membrane guanylate cyclase activity. Membranes (37 μ g of membrane protein per reaction) were prepared from hippocampus and allowed to react for 4 min with the indicated concentrations of sAPP. All values represent the mean $\pm$ S.E.M. of triplicate determinations (* $P < 0.05$ versus control saline).

inhibitor, ZnPPiX. This treatment had no effect on the sAPP-evoked depression of $[Ca^{2+}]_i$ (Figure 1b).

To confirm more directly the indications provided by measurements of $[Ca^{2+}]_i$, we assayed cGMP responses to sAPP. Primary cultures from rat hippocampus were pretreated with 100 μ M N-Arg or 10 μ M ZnPPiX and compared with untreated cultures in their responses to sAPP. Consistent with the assays of $[Ca^{2+}]_i$, neither N-Arg nor ZnPPiX affected the elevation of cGMP by sAPP; cGMP levels (pg/mg of protein) after a 3 min incubation were as follows: control, 0.794 ± 0.528 ; sAPP (200 pM), 2.80 ± 0.517 ; sAPP and N-Arg, 2.82 ± 0.418 ; sAPP and ZnPPiX, 2.96 ± 0.475 . Together these data suggest that soluble guanylate cyclase is not involved in the elevation of cGMP by sAPP.

Lack of involvement of soluble guanylate cyclase implied the stimulation of a membrane-associated cyclase. To test this possibility, we prepared membranes from neonatal rat brain and assayed the generation of cGMP from exogenous GTP. In other systems, membrane guanylate cyclases have been shown to be affected by sodium orthovanadate, reducing agents and an allosteric modulation by ATP. These variables were tested

independently in the presence or absence of sAPP. A stimulation of cyclase activity by sAPP was apparent only in the presence of 1 mM ATP (results not shown); therefore ATP was included in all the subsequent assays. Neither sodium orthovanadate nor reducing agents had any effect on the stimulation of cyclase activity by sAPP. Kinetic characterizations were then performed, which indicated that basal and stimulated guanylate cyclase activities remained linear within 6 min; most subsequent assays were performed within 4 min. The stimulation of cyclase activity by sAPP was also characterized with respect to pH and was found to have an optimum pH of 7.0 (Figure 2a). Intuitively, this value seems suitable for the relatively acidic nature of the neuronal cytoplasm [18]. This variable was also incorporated into subsequent assays. Stimulation of cyclase activity by sAPP reached half its maximum at approx. 1 nM (Figure 2b). This is a notably higher concentration than that required to reach a similar stimulation of cGMP formation in intact cells [8], suggesting that preparation of membranes may either remove an unknown soluble modulator of the guanylate cyclase or detrimentally affect its lipid environment in such a way as to compromise the stimulation.

DISCUSSION

Previous findings suggested that the potent effects of sAPP on neuronal physiology and viability are mediated, at least in part, by an activation cGMP-dependent protein kinase. We have extended this line of investigation by determining the class of guanylate cyclase stimulated by sAPP. Neither the depression of $[Ca^{2+}]_i$ nor the elevation of cGMP by sAPP was affected by pharmacological suppression of the second messengers that activate soluble (cytosolic) guanylate cyclases. More importantly, sAPP was able to stimulate an elevation of cGMP in purified membranes.

All membrane-associated guanylate cyclases isolated to date are components of a single polypeptide that spans the plasma-membrane and acts as the receptor for an extracellular ligand. Therefore one implication of the present study is that a signal-transducing receptor for sAPP may exist that contains a cyclase domain similar to that present in other receptors of this class. Specific purification strategies based on the properties of these receptors have been consistently successful for the isolation and cloning of such receptors [13].

Another implication of these findings concerns the spatial distribution of the sAPP-stimulated cGMP signal. The elevation of cGMP by sAPP in intact cells is always within 300% [8]. This seems modest compared with responses to agonists that utilize the soluble guanylate cyclase abundant in neuronal cytosol. However, spatial limitation of the cGMP generated in response

to sAPP to subcellular regions near the membrane may make the signal more effective in modulating membrane events [19]. Candidates for such a membrane-localized effect may include ion channels. We have recently determined that sAPP rapidly activates a large-conductance potassium channel in hippocampal neurons through a cGMP-dependent mechanism (K. Furukawa, S. W. Barger, E. M. Blalock and M. P. Mattson, unpublished work). This and other membrane-localized events could be involved in acute modulations of neuronal physiology by sAPP, predicting a role for sAPP (and its presumed cyclase-containing receptor) in physiological plasticity. Such a role is already suggested by the impairment of memory acquisition by APP mutation [20] or APP antibodies [21].

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