Transmembrane Signal Transduction and ACTH-induced Excessive Grooming in the Rat

WILLEM HENDRIK GISPEN

Division of Molecular Neurobiology
Rudolf Magnus Institute for Pharmacology
and
Institute of Molecular Biology and Medical Biotechnology
3584 CH Utrecht, the Netherlands

INTRODUCTION

In this paper I shall review some of the neurochemical events that possibly underlie ACTH-induced excessive grooming in the rat. In previous reviews I have documented this behavioral reponse to ACTH and congeners in great detail.[1,2] The response is readily seen following intracranial administration of ACTH and can be recorded using a time sampling method yielding quantitative information on occurrence and duration of grooming bouts.[3] As studied by many authors, the grooming bout consists of a rather fixed pattern of motor acts which I refer to collectively as grooming behavior. In view of some of the evidence also presented at this meeting, it is increasingly clear that different behavioral elements seen as part of the grooming bout may originate from different neural substrates and possibly may result from different ACTH brain interactions. As in most of the studies in which both behavioral and neurochemical aspects are monitored, the behavioral response to ACTH is merely measured in terms of frequency and duration of grooming as such and not in terms of the display of various behavioral elements constituting grooming behavior. I cannot describe any neurochemical event in relation to the display of a particular grooming element. Nonetheless, as will be discussed below, by looking at grooming as a whole much progress has been made in our understanding of how ACTH acts in the brain.

In the search for the neurochemical mechanism of action of ACTH a major problem has been the lack of information on stereo-specific, saturable binding sites for this peptide.[4] Although the brain has networks of neurons which express pro-opiomelanocortin (POMC), the precursor for peptides of the ACTH family, and ACTH peptides have been found in brain and liquor,[5] the absence of information on a specific ACTH receptor is surprising and puzzling. Previously it was reported that ACTH-like peptides might act as partially agonist-antagonist at central opiate receptors.[6,7] However, the high concentrations of peptides necessary to displace the labeled opiate from its receptor have called into question the physiological importance of these findings. Although opiate antagonists have been shown to block ACTH-induced excessive grooming,[8,9] I still maintain that the opiate-sensitive component in grooming behavior is a site which is distal to the primary site at which ACTH acts to initiate the grooming bout.[10]

Despite the drawback that presently no information is available on the first interaction of the peptide with neural cells, much information has been gained concerning

ACTH-sensitive processes in neuronal cell membranes normally serving a role in receptor-mediated transmembrane signal processing.[11] Both in the adrenal cortex cell[12] and in brain tissue,[11] ACTH may activate or modulate at least two different signal transduction systems, *i.e.*, the receptor-activated production of cAMP and the receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) followed by the production of diacyglycerol (DG) and D-myoinositol 1,4,5-trisphosphate (IP_3; see below). As the structure activity of ACTH in excessive grooming is very similar to that observed in the modulation of the receptor-mediated poly-PI response (see below), the significance of this response for ACTH-induced excessive grooming will be assessed in detail.

RECEPTOR-MEDIATED POLYPHOSPHOINOSITIDE HYDROLYSIS

Activation of various hormone and neurotransmitter receptors results in phosphodiesteratic degradation by a phospholipase C (PLC) of PIP_2 to IP_3 and DG. IP_3 can be either first phosphorylated or be directly degraded to myoinositol. DG is phosphorylated to phosphatidate and then, through a liponucleotide intermediate, converted to phosphatidylinositol (PI). In two phosphorylation steps, PI is converted via phosphatidylinositol 4-phosphate (PIP) to PIP_2 (FIG. 1).[13,14] The relatively low content of PIP_2 and the transient nature of subsequent increases in IP_3, D-myoinositol 1,4-bisphosphate, and D-myoinositol 1-monophosphate, and decreases in PIP_2, PIP, and PI suggest that PIP_2 is replenished after its breakdown by PDE. Such replenishment appears to provide a mechanism to control the inositide response.[15]

Both initial PDE products have a second messenger function in the cell (FIG. 1). IP_3 or a metabolite thereof can mobilize Ca^{2+} from putative endoplasmic reticulum stores.[16,17] DG can activate protein kinase C (PKC). The activity of PKC is dependent on the presence of an acidic phospholipid such as phosphatidylserine (PS) and is calcium sensitive. DG enhances the calcium sensitivity to PKC to the concentration normally present in an ionized form in the cell.[18]

The DG derivative dioctanoylglycerol (DOG) or the phorbol diester phosphoryl 12,13-dibutyrate (PDB) mimic DG in this respect, but the latter is about 1000-fold more potent than DG and has hence been a useful tool to investigate PKC.[18] In various cell types protein substrates of PKC have been identified, although the function of most of them cannot as yet be defined. In brain, two phosphoproteins in synaptic plasma membranes (SPM) have been characterized as a substrate of PKC, *i.e.*, an 87 kDa protein[19] and the neuron-specific phosphoprotein B-50.[20] It is believed that these substrate proteins may mediate the function of PKC in the neuronal response to enhanced hydrolysis of PIP_2.

THE ROLE OF THE PKC SUBSTRATE PROTEIN B-50 IN THE POLYPHOSPHOINOSITIDE RESPONSE

The phosphoprotein B-50 (M_r 48 kDa, pI 4.5) is neuron-specific and in adult rat brain is predominantly found in presynaptic terminals, presumably associated with the inner side of the plasma membrane and vesicle membranes.[21-23] In view of the similarities in apparent M_r, pI, substrate specificity, metal requirements, peptide maps, sensitivity to modulators, phospholipids, and protease treatment,[20,24] it was concluded that B-50

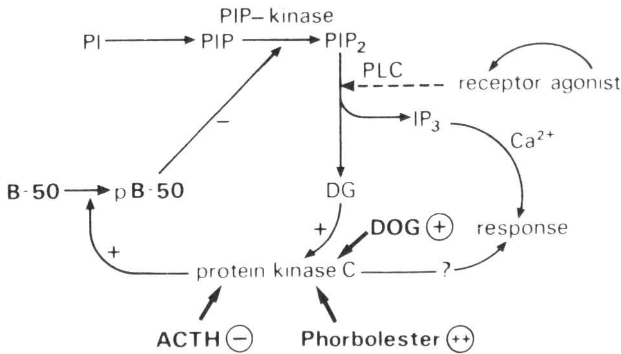

FIGURE 1. Model of the regulatory role of B-50 in receptor-mediated polyphosphoinositide hydrolysis in brain.

protein kinase is very similar if not identical to PKC. This is further substantiated by recent findings indicating that DOG and phorbol esters stimulate the phosphorylation of B-50 in synaptic plasma membranes.[25,26] These data link B-50 via its B-50 kinase/PKC to the role that enhanced production of DG plays in synaptic membrane function (see FIG. 1).

Under a variety of conditions, it was found that there is a reciprocal relationship between the degree of phosphorylation of B-50 and the activity of PIP kinase (for review see ref. 25). Partially purified PIP kinase from rat brain cytosol was tested in the presence of added purified B-50 preparations, which differed in their degree of phosphorylation. In such a reconstituted system, conditions were found under which phosphorylated B-50 proteins reduced PIP kinase activity while dephosphorylated B-50 did not.[27] It was suggested, therefore, that the B-50 protein may be an endogenous modulator of PIP kinase activity in rat SPM (FIG. 1).

As discussed above, activation of certain receptors is associated with enhanced hydrolysis of PIP_2. The degraded polyphosphoinositide (PPI) is replenished via PI and PIP, ultimately involving PIP kinase.[28] As enhanced phosphorylation of B-50 is accompanied by a decrease in the activity of PIP kinase, it has been proposed that the sequence DG production, PKC activation, B-50 phosphorylation, PIP kinase inhibition may represent a negative feedback control mechanism in the receptor-mediated hydrolysis of inositol phospholipid (FIG. 1).[29] In fact, several authors have recently shown that direct activation of PKC by means of phorbol esters reduces the production of inositol phosphates in response to muscarinic receptor activation.[30-32]

The proposed feedback role of B-50 in the inositol lipid response was tested in hippocampal slices using the behaviorally active $ACTH_{1-16}$-NH_2 to inhibit and PDB to stimulate PKC. We confirmed the results of Labarca *et al.*[30] and showed that preincubation of hippocampal slices with PDB diminished subsequent receptor-mediated hydrolysis of PIP_2 by incubation with carbamylcholine. More importantly, it was shown that preincubation with $ACTH_{1-16}$-NH_2, known to inhibit PKC, counteracted the phorbol effect on receptor-mediated PIP_2 hydrolysis. Thus, under appropriate conditions ACTH-like peptides may modulate synaptic transmembrane signal transduction through a reduction of the negative feedback role of the PKC–B-50–PIP kinase loop.[33]

ARE CHANGES IN B-50 PHOSPHORYLATION RELATED TO THE EXPRESSION OF GROOMING?

Some 10 years ago, we reported some preliminary findings that pointed to changes in the post hoc *in vitro* phosphorylation of several SPM proteins following prior *in vivo* administration of $ACTH_{1-24}$.[34] In FIG. 2, the effect on *in vivo* grooming and *in vitro* B-50 phosphorylation is presented. As expected the intraventricular administration of $ACTH_{1-24}$ induced excessive grooming in a dose-dependent manner. The doses used were 3, 30, 300, and 3000 ng per 3 μl i.c.v. and the behavior was analyzed by a time sampling procedure between 10 and 30 min following the i.c.v. injection. Immediately after termination of the behavioral recording the rats were killed by decapitation, their subcortical brain tissue was dissected out, and light synaptic plasma membranes were isolated. The endogenous phosphorylation of SPM proteins was studied by addition of [γ-^{32}P]ATP and the incorporation of phosphates into individual proteins was quantified by autoradiographic scanning of SDS-PAGE gels. It was found that several low molecular weight proteins showed an enhanced phosphorylation following ACTH administration *in vivo.* Although in the original report the quantification of an 18 kDa protein is presented, the response of the 48 kDa protein (B-50) is identical.[34] We discussed the difficulty of studying post hoc phosphorylation following an experiment like ACTH-induced grooming. Indeed, a variety of factors may have contributed to the observed change in phosphorylation. Within the context of this paper, the question is whether the observed change in phosphorylation is related to the grooming activity per se or to the interaction of ACTH with certain populations of neurons involved in the expression of the grooming response. Our conclusion at the time was that since *in vitro* addition of $ACTH_{1-24}$ leads to changes in the phosphorylation of the same low molecular weight bands, the latter possibility seemed the more likely.

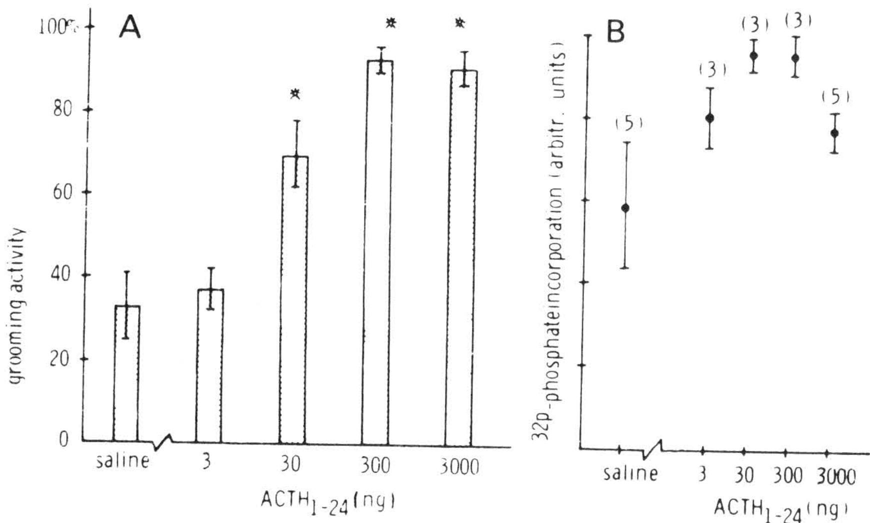

FIGURE 2. **(A)** ACTH-induced excessive grooming in the rat. Mean ± SEM, $n = 4$, * $p < 0.05$ (t-test, two-tailed). **(B)** [^{32}P] incorporation into B-50 in SPM after *in vivo* administration of $ACTH_{1-24}$ (30 min; numbers in parentheses represent number of experiments).

Our neurochemical tools in the analysis of B-50 phosphorylation have improved tremendously yet we never went back and repeated this experiment. Thus, although presently more accurate data could be collected addressing this issue more properly, I am convinced at least as far as B-50 is concerned that the conclusion that *in vivo* administration of ACTH may lead to a change in the endogenous phosphorylation as measured in a post hoc assay is valid, for we and others have employed this approach extensively in a variety of experimental designs.

Our interest in the function of the B-50 phosphoprotein originates from our observation that ACTH and congeners *in vitro* inhibit the phosphorylation of this protein by reducing the activity of PKC.[35,36] In a series of experiments the peptide structure requirements for inducing excessive grooming *in vivo* and for inhibiting PKC in SPM *in vitro* were studied. As can be seen in FIG. 3, there is a striking similarity in structural requirements. For instance, the full sequence $ACTH_{1-24}$ is the most active whereas the constituting fragments $ACTH_{1-10}$ and $ACTH_{11-24}$ are virtually inactive in both assays. Even the equimolar combination of $ACTH_{1-10}$ and $ACTH_{11-24}$ is without effect on grooming and PKC. To my knowledge this is one of the best examples of a correlation between a behavioral and a neurochemical activity of a given peptide. I have interpreted these data to underscore the importance of the stereo-confirmation on the effect of the ACTH molecule on the brain. Furthermore, these data show that the presumed ACTH-sensitive sites in the brain *in situ* respond to the information encoded in the ACTH peptide in the same way as the membrane-bound enzyme PKC does when

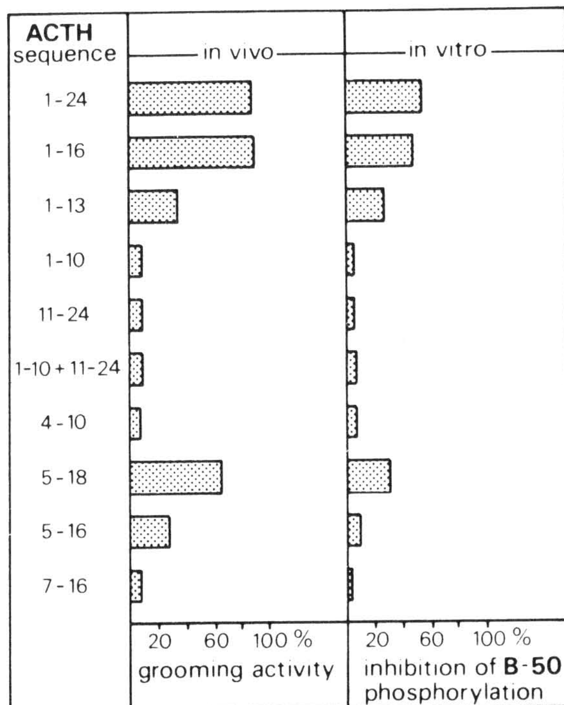

FIGURE 3. Comparison of structural requirements of ACTH for inducing excessive grooming and inhibition of B-50 phosphorylation.

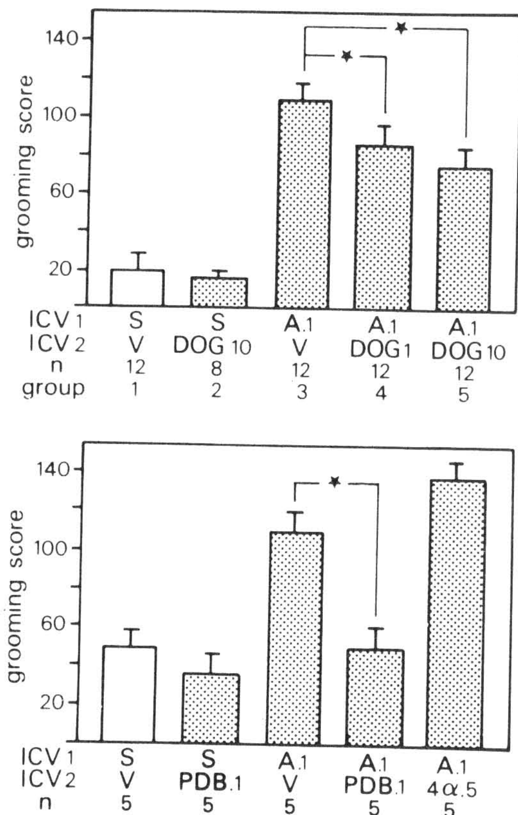

FIGURE 4. The effect of 1,2-dioctanoyl glycerol (*DOG*, top panel) and 4β-phorbol 12,13-dibutyrate (*PDB*, bottom panel) on ACTH-induced excessive grooming: *ICV1*, *ICV2*, first and second intracerebroventricular injection; *S*, saline; *V*, 0.5% ethanol in saline; *A.1*, 0.1 μg $ACTH_{1-24}$ in saline; *DOG 1*, *DOG 10*, 1 or 10 μg DOG in 0.5% ethanol in saline; *PDB.1*, 0.1 μg PDB in 0.5% ethanol in saline; *4α.5*, 0.5 μg 4α-phorbol in 0.5% ethanol in saline; *n* = number of rats; *bars*, mean ± SEM; *asterisks*, significantly different from group 3 ($p < 0.05$).

measured *in vitro*, again suggesting that the latter may be involved in the mechanism by which ACTH induces grooming.

If inhibition of PKC is part of the mechanism by which ACTH induces grooming, it was reasoned that concomitant stimulation of this enzyme by phorbol diesters should reduce this behavioral response to ACTH. Rats were first given $ACTH_{1-24}$ (i.c.v. 0.1 μg/3 μl) followed by either 0.5% ethanol in saline (vehicle) or DOG, PDB, PMA, or 4α-PDB, respectively. In order to compare the relative potency of the three phorbol esters to DOG, equimolar amounts to 1 μg DOG were used. As can be seen from FIG. 4, the biochemically nonactive 4α-PDB did not reduce the grooming in response to ACTH. The potency order by which the other compounds inhibit ACTH-induced excessive grooming is PDB > PMA > DOG. This potency order is similar to that found for the activation of PKC by this compound in SPM.

CONCLUDING REMARKS

Although the evidence on the involvement of PKC inhibition in ACTH-induced excessive grooming in increasing, great caution is still warranted in interpreting the significance of our findings.

First of all, most of our work concerned broken cell preparations rather than intact cells or circuits. Only recently have we been able to demonstrate effects of ACTH in intact systems that support the role in altering the negative feedback in the PPI response as was suggested from the data obtained in *in vitro* studies.[33,37] However, even if the peptide was indeed operative via PKC inhibition, how is this accomplished across the membrane as PKC is localized at the inner side of the cell envelope? Previously, Gysin and Schwyzer[38] have discussed the possibility that the ACTH-like peptides might influence membrane function by interactions other than classical receptor activation. The NH_2 terminus of ACTH known to contain all biological activity of the molecule has been shown to form an amphipathic helix[39] that could be inserted into the membrane bilayer upon proper interaction of the positively charged region of the peptide with negatively charged domains in the cell membranes.[38] Such an insertion might bring the biologically important NH_2 terminus in the close vicinity of membrane-bound PKC.

Secondly, the doses at which the peptide inhibits PKC in SPM are considerably larger (IC_{50} 10^{-7} M) than those necessary to activate the signal transduction pathways in the peripheral target cell.[12] This and other issues have led us to propose that the suggested mechanism of action of ACTH on SPM protein phosphorylation would only be of functional significance in brain regions rich in projections of neurons that produce melanocortins, for in such peptidergic synaptic clefts the peptide concentration would be sufficiently high to allow for the train of events depicted in FIG. 1.

Given such a regional specificity of the neurochemical effect of ACTH, what region do we know of that contains PKC and B-50, peptidergic terminals, and is part of the neural substrate underlying behavioral activity of ACTH?

If grooming behavior is considered as a behavioral response to ACTH then the periaqueductal gray is of singular importance. Following reports on opiate effects of implantation of extremely high doses of ACTH into the periaqueductal gray[40] and on the role of periaqueductal gray in bombesin-induced grooming in rats,[41] Spruijt *et al.*[42] concluded that the PAG is essential for the display of ACTH-induced grooming and suggested that this structure was the primary target for the peptide to act upon. Other transmitter systems such as those containing DA and GABA would modulate the output of the PAG, *i.e.*, ACTH-induced excessive grooming.[42] Furthermore, Oestreicher *et al.*[43] demonstrated that the PAG contains relatively high concentrations of B-50. Finally, it is well-established that this brain region receives peptidergic projections containing peptides of the melanocortin family.[44] We are therefore currently studying the significance of ACTH modulation of synaptic transmembrane signal transduction systems in the PAG in relation to induction of excessive grooming. Thus, in addition to the behavioral study of the significance and structure of peptide-induced grooming per se, my laboratory has also used this behavioral response to ACTH as a reliable and relatively simple model system to unravel some of the molecular aspects underlying the behavioral activity of ACTH.

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