REGULATION OF NEUROTRANSMITTER RESPONSES IN THE CENTRAL NERVOUS SYSTEM (U)
TEXAS UNIV MEDICAL SCHOOL AT HOUSTON
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UNCLASSIFIED
The aim of this study was to characterize the mechanism whereby activation of GABA receptors augments (modulates) receptor-mediated second messenger production in brain. Using rat brain slices it was found that the ability of GABA and norepinephrine agonists to augment the cyclic AMP response to isoproterenol was greatly diminished in the presence of EGTA or quinacrine. Likewise, this response was attenuated in the brains of animals treated chronically with corticosterone. Moreover, it was discovered that phorbol esters augment the second messenger response in a manner similar to the GABA and adrenergic agonists. Phosphorylation studies revealed that exposure of the brain tissue to phorbol esters leads to the phosphorylation of a constituent that resembles the inhibitory guanine nucleotide binding protein. These findings suggest that the modulation of brain neurotransmitter responses may be mediated by activation of phospholipase A2 and/or protein kinase C, and that the critical factor in the modulatory response may be the phosphorylation of a protein that regulates adenylate cyclase activity.
The aim of this research program was to characterize and define the manner in which neurotransmitter receptor systems are modulated and modified in the central nervous system. The primary emphasis of the research was on characterizing the manner in which γ-aminobutyric acid (GABA), through an interaction with GABA₆ receptors, augments second messenger responses to other transmitter agents, in particular norepinephrine. Given the hypothesis that the modulation of neurotransmitter responses may be the primary mechanism for the subtle manipulation of central nervous system activity, a better understanding of this process may make possible the development of drugs capable of enhancing or reducing central nervous system responses. In theory, such agents may be useful in enhancing cognition, alertness, manual dexterity, and a variety of bodily functions that are under the tonic control of the nervous system.

The idea for this study was generated by the discovery that GABA, through an interaction at GABA₆ receptors, greatly augments the response obtained in the presence of agents known to directly stimulate the production of cyclic AMP. Of particular interest was the discovery that GABA₆ agonists such as baclofen augment the response to norepinephrine, a neurotransmitter agent known to be important for regulating a variety of central nervous system functions. During the fifteen months of support, a significant amount of progress was made in defining further the mechanisms underlining the relationship between the GABA₆ receptor system and those associated with second messenger production. Thus, it was found that the
baclofen-induced augmentation of cyclic AMP accumulation is a calcium-dependent phenomenon since incubation of the brain slices with EGTA, a substance that reduces extracellular concentrations of calcium, greatly diminished the augmenting response to baclofen while leaving unaltered the receptor response to a β-adrenergic agonist (isoproterenol). This finding suggests that GABAₐ agonists facilitate the entry of calcium into the cell which in turn is important for mediating the augmenting response.

Similar data were obtained in a series of experiments aimed at examining the influence of α₁-adrenergic receptor agonists on isoproterenol-stimulated cyclic AMP accumulation. In these studies it was found that the α₁-adrenergic agonists, like baclofen, augment the second messenger response. Moreover, the augmenting action of the α₁-agonists was also found to be a calcium-dependent phenomenon. This discovery indicates that receptor modulation, regardless of the agents involved, has similar characteristics at a biochemical level.

Given the suggestion that calcium is an obligatory factor with regard to the augmenting response, experiments were performed to study the relationship between various calcium-dependent processes and the GABAₐ and α₁-adrenergic receptor systems. Several years ago it was suggested by others that the calcium-dependent enzyme phospholipase A₂ (PLA₂) might be involved in cyclic AMP production, suggesting that an examination of this enzyme in the GABAₐ modulatory phenomenon was warranted. To this end, the effect of quinacine, a nonselective inhibitor of phospholipase A₂, was studied on the augmentation phenomenon. The results indicated that quinacine completely abolished the augmenting response to both GABAₐ and α₁-adrenergic agonists, supporting the notion that phospholipase A₂ may be an important link in the augmenting response. More direct evidence for this conclusion was provided by the finding that corticosterone
administration reduces the augmenting response as well. Thus, it has been established that corticosterone administration stimulates production of endogenous peptides (e.g. macrocortin) which inhibits PLA₂. Assuming that macrocortin is produced in brain, the present findings can be taken as further evidence that phospholipase A₂ activity is involved in mediating the augmenting response to both GABA₉ and α₂-adrenergic agonists.

A major role for PLA₂ is to catalyze the conversion of membrane phospholipids to arachidonic acid and lysophospholipid. Arachidonic acid is, in turn, metabolized to a variety of biologically active substances, such as the prostaglandins. To determine whether the production of prostaglandins is the key factor in the modulating response, experiments were performed with a variety of substances known to inhibit arachidonic acid metabolism (indomethacin, ibuprofen, nordihydroguaiaretic acid). The results of these experiments demonstrated that none of these substances selectively influence the augmenting response, indicating that arachidonic acid itself, or lysophospholipid, is important for regulating the augmenting action of GABA₉ and α₂-adrenergic agonists.

In a separate series of experiments, the effect of phorbol esters was examined on the cyclic AMP generating response in brain slices. The results indicated that tumor-promoting phorbol esters, substances known to directly activate protein kinase C, a calcium-dependent enzyme, were, like baclofen and α₂-adrenergic agonists, capable of augmenting second messenger responses to the β-adrenergic agonist. This finding indicates that, like PLA₂, protein kinase C may be an important enzyme in regulating the modulatory response. Additional experiments revealed that neither GABA₉ nor α₂-adrenergic agonists have any effect on phospholipase C, an enzyme that is normally activated prior to the in vivo stimulation of protein kinase C. Given the suggestion that protein kinase C might contribute to the modulatory response, this finding made it difficult to propose a model
whereby this enzyme could be influenced by baclofen and α₂-agonists. Recently, however, it is been found that a variety of fatty acids, including arachidonic acid, are capable of activating protein kinase C. This discovery may prove to be important in providing a link between the results with PLA₂ and the phorbol esters.

Thus, based on the present data it would appear that activation of either GABA₉ or α₂-adrenergic receptors leads to the entry of calcium ions and to the stimulation of PLA₂. When the PLA₂ is activated it catalyzes the formation of arachidonic acid which, in turn, is capable of stimulating protein kinase C. Like other kinases, it is known that protein kinase C phosphorylates intercellular proteins, modifying their activity. Thus, the augmenting response is presumably secondary to the phosphorylation of some substrate that is important for regulating cyclic AMP accumulation. One possible candidate is the inhibitory guanine nucleotide binding protein (Nᵢ) since it has been found in platelets that stimulation of protein kinase C leads to the phosphorylation of this substance. A similar action in brain could explain the augmenting response since Nᵢ exerts an inhibitory effect on adenylate cyclase, the enzyme responsible for the formation of cyclic AMP. Thus, by phosphorylating Nᵢ and reducing its influence on adenylate cyclase, it would be expected that agents stimulating cyclic AMP formation would yield a more dramatic increase in the production of this second messenger. Evidence that this may occur in the brain was provided by some of our more recent experiments aimed at determining the phosphorylation pattern obtained in brain tissue following exposure of the brain slice to phorbol esters. As shown in these studies, a band of phosphorylation was found in an area corresponding to where Nᵢ should be present, consistent with the notion that this protein may serve as a substrate for protein kinase C in brain. Accordingly, it appears quite possible that the modulatory response obtained with GABA₉ and α₂-adrenergic agonists is due to the ability of these substances to lead to
a reduction in the inhibitory activity of a guanine nucleotide binding protein coupled to adenylate cyclase.

In a separate series of experiments we have undertaken an examination of the developmental profile of the augmenting response to GABA$_b$ agonists. The results indicate that the augmentation is detectable within a few days after birth, increasing dramatically during the first week of development. Interestingly, unlike most neurotransmitter receptor systems, the augmenting response diminishes thereafter, such that by three weeks of age it has returned to adult levels. By studying the characteristics of the response in the developing brain when augmentation is most magnified, it may be possible to obtain more precise information about the mechanism of this response. Of particular interest is our recent finding that protein kinase C has a developmental pattern similar to that found for the augmenting response, providing additional support for our hypothesis that protein kinase C may be a crucial enzyme for regulating neuromodulation in brain.

Given the ultimate goal of developing novel pharmacological agents for manipulating central nervous system function, the discovery of a biochemical response to a neuromodulator can be considered a major step in that direction. Thus, up to now, neuromodulatory substances were difficult to study since there was no simple biochemical method for detecting the response to such agents. In addition to providing new insights into the mechanism of neuromodulation, the data from our studies yields a simple method that can be used for the design, testing and development of such agents.

Using this, and other approaches, attempts were also made to identify novel GABA$_b$ receptor agonists and antagonists. Indeed, the absence of GABA$_b$ receptor antagonists has been a major hinderance into defining the
behavioral and physiological importance of this neuromodulating system. During the course of our studies we found one agent (2-butyl-GABA) that displays some antagonist properties in vivo, although its potency and selectivity in this regard appear to be insufficient to warrant further investigation. However, based on this finding we proposed to study the action of 2-substituted baclofen analogs. In collaboration with a chemist, we were able to synthesize sufficient quantities of 2-butyl-baclofen to test in our system. However, the results with this agent were disappointing, with 2-butyl-baclofen being virtually inactive as a GABA antagonist either in vitro or in vivo under our assay conditions. Nevertheless, we believe that our initial findings with the 2-butyl analogs of GABA may be an important lead in the development of selective, potent and specific GABA antagonist either in vitro or in vivo under our assay conditions. This represents an important line of investigation since, without such an agent, it will never be possible to totally understand the importance of the GABA system in regulating normal central nervous system function.

In summary, during the funding period significant progress was made toward understanding the biochemical characteristics of GABA and \( \alpha_2 \)-adrenergic receptor-mediated modulatory responses in brain. Given the fact that the underlying mechanism responsible for the augmentation mediated by these two systems appears to be similar, it is conceivable that the present data will apply to neuromodulation in general, and therefore have applicability beyond the narrow scope of this project. Based on these findings, it would appear that modulation of neurotransmitter responses involves a number of separate elements including PLA, protein kinase C, arachidonic acid, and guanine nucleotide binding proteins. Given this number of targets, it seems likely that novel therapeutic agents can be developed that may be capable of influencing the modulatory response. Because of the likelihood that a substance capable of influencing neuromodulation will have a more subtle effect on neurotransmitter
function, it seems possible that such an agent will have a great potential for manipulating central nervous system activity both in normal and dysfunctioning individuals.

MANUSCRIPTS PUBLISHED DURING THE SUPPORT OF THIS PROGRAM (copies enclosed):


Regulation of Neurotransmitter responses in the Central Nervous System (unclassified)

Salvatore J. Enna

The aim of this study was to characterize the mechanism whereby activation of \( \gamma \)-aminobutyric acid \(_B\) (GABA\(_B\)) receptors augments (modulates) receptor-mediated second messenger production in brain. Using rat brain slices it was found that the ability of GABA\(_B\) and \( \alpha \)-adrenergic agonists to augment the cyclic AMP response to isoproterenol was greatly diminished in the presence of EGTA or quinacrine. Likewise, this response was attenuated in the brains of animals treated chronically with corticosterone. Moreover, it was discovered that phorbol esters augments the second messenger response in a manner similar to the GABA\(_B\) and \( \alpha \)-adrenergic agonists. Phosphorylation studies revealed that exposure of the brain tissue to phorbol esters leads to the phosphorylation of a constituent that resembles the inhibitory guanine nucleotide binding protein. These findings suggest that the modulation of brain neurotransmitter responses may be mediated by activation of phospholipase A, and/or protein kinase C, and that the critical factor in the modulatory response may be the phosphorylation of a protein that regulates adenylate cyclase activity.
Receptor regulation: evidence for a relationship between phospholipid metabolism and neurotransmitter receptor-mediated cAMP formation in brain

S. J. Enna and E. W. Karbon

Neurotransmitter receptor responsiveness is regulated by a variety of factors. One of these appears to be through an association between the neurotransmitter receptor-coupled effector system and receptors for other substances that serve to modify, rather than directly activate or inhibit, the second messenger response. S. J. Enna and E. W. Karbon review data indicating that the regulatory influence of GABA<sub>B</sub> and α-adrenergic agonists on receptor-stimulated cAMP accumulation in brain slices may be mediated by calcium-associated enzymes, in particular phospholipase A<sub>2</sub> and protein kinase C. A model is proposed linking these two enzyme systems and an hypothesis presented that this regulatory action may be important for controlling receptor sensitivity.

Neurotransmitter receptors play a fundamental role in relaying information within and between the central and peripheral nervous systems and are therefore primary targets for pharmacological agents. Most drugs of this type influence receptor function directly by attaching to the receptor recognition site, mimicking or blocking the action of the endogenous ligand. However, it is becoming increasingly apparent that synaptic activity is not an all or none phenomenon, but is subject to regulatory influences that continuously maintain a certain level of functioning between extremes. Given this context, it would seem that direct stimulation or inhibition of neurotransmitter receptors may not always be the ideal approach for re-establishing homeostasis in a diseased system. Indeed, many of the limitations associated with the use of current medications appear due to the desensitization or supersensitivity that result from prolonged and persistent activation or blockade of receptor sites. Thus, it is conceivable that agents influencing the regulatory mechanisms associated with receptor function, rather than directly interacting with the receptor itself, may in some cases have more subtle and therefore more therapeutically useful effects. Inasmuch as the development of such drugs requires a detailed understanding of the mechanisms associated with receptor regulation, the number of studies on this topic has increased in recent years. The present review highlights one aspect of this work.

Regulation of cAMP production

Neurotransmitter receptor activation modifies cellular activity through a coupling between the recognition site and an effector mechanism. In some cases the recognition site is directly associated with an ion channel whereas in others the effector is a membrane-associated enzyme that generates the production of a second messenger. One of the most intensively investigated second messenger systems is that associated with adenylate cyclase. Thus, activation of certain neurotransmitter receptor recognition sites enhances adenylate cyclase activity, catalyzing the production of cAMP from ATP. Other neurotransmitter receptors, such as cholinergic muscarinic, are negatively coupled to this enzyme such that their stimulation reduces cyclase activity. In both cases the receptor signal is transmitted to the catalytic unit of adenylate cyclase through a membrane-bound guanine nucleotide binding protein (G<sub>i</sub>), with a stimulatory protein (G<sub>s</sub>) activating, and an inhibitory protein (G<sub>i</sub>) reducing, cAMP production. Once formed, cAMP activates a kinase that catalyses the phosphorylation of select proteins resulting in a modification in cellular activity. The second messenger is subsequently converted to 5′-AMP by a family of phosphodiesterases. Thus, intracellular levels of cAMP are regulated by the coordinated interaction of a variety of cellular components, with the modification of any one having the potential to influence receptor function.

Some receptors do not appear to be directly coupled to adenylate cyclase, since their activation alone does not modify cAMP production. However, cAMP accumulation is augmented dramatically when these sites are stimulated simultaneously with those that are positively coupled to G<sub>s</sub> and G<sub>i</sub> agonists, such as arginine vasopressin, which by itself has no effect on cAMP accumulation in pituitary corticotrophs, potentiates the second messenger response to corticotropin-releasing factor in these cells. Moreover, activation of α-adrenergic receptors in brain slices augments β-adrenergic receptor-stimulated cAMP accumulation, as does γ-aminobutyric acid (GABA) through an action at GABA<sub>B</sub> receptors (Fig. 1). Because neither α-adrenergic nor GABA<sub>B</sub> agonists influence cAMP accumulation when the brain slice is exposed to them alone, it would appear that their receptors serve to regulate, rather than mediate, second messenger production. Curiously, exposure of brain membrane fragments, rather than intact tissue, to GABA<sub>B</sub> and α-adrenergic agonists reduces adenylate cyclase activity, suggesting that in broken cells they activate a receptor that...
may be negatively coupled to the enzyme. It remains to be determined whether the inhibitory action observed in membranes and the augmenting response in slices are mediated by the same receptor and which, if either, represents the normal physiological response. Inasmuch as the brain slice appears to more closely approximate the in vivo situation, this report will concentrate on findings obtained with this preparation.

Phospholipase C

Studies with brain tissue have revealed that the augmenting response to GABA_B and o-adrenergic agents is observed with virtually all substances known to stimulate receptors that are positively coupled to adenylyl cyclase. This includes receptors for adenosine, histamine, 1-adrenergic agonists and vasoactive intestinal peptide. Experiments aimed at defining the mechanism by which these agents augment cAMP accumulation indicate that calcium ion is required since the augmentation is abolished in the presence of EGTA, a chelator of this ion. Among other possibilities, this discovery suggests the involvement of calcium-dependent enzymes in the augmenting response. Inasmuch as phospholipid metabolism is regulated in part by such enzymes, and since studies suggest that phospholipids and their metabolites can influence cyclic nucleotide formation, it is conceivable that the augmenting effect of GABA_B and o-adrenergic agonists may be associated with phospholipid turnover.

Studies have been undertaken to determine whether phospholipase A_2 (PLA_2), a calcium-activated enzyme that catalyses the release of arachidonic acid from membrane phospholipids, contributes to the cAMP-augmenting response. Indeed, mepracine, a non-selective inhibitor of PLA_2, has been found to reduce the augmenting response to o-adrenergic and GABA_B agonists at concentrations that fail to influence directly receptor-stimulated cAMP accumulation. Moreover, chronic administration of corticosterone, a hormone that stimulates the production of an endogenous inhibitor of PLA_2, diminishes the augmenting response. These data support the notion that phospholipid metabolism, in particular that associated with PLA_2, may be an important contributory factor in the augmenting response. Furthermore, the finding that inhibitors of cyclooxygenase and phospholipase C are effective in reducing the augmenting response suggests that a calcium function is involved in the augmenting activity, either as a stimulator or an inhibitor of PLA_2 activity. It is conceivable that phospholipid metabolites themselves, but not their purine or pyrimidine bases, act as second messengers to mediate the augmenting response to o-adrenergic agents.

Phospholipase C

Another enzyme associated with phospholipid metabolism and transmitter receptors is phospholipase C (PLC). Certain neurotransmitters stimulate this enzyme, which in turn catalyses the conversion of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate (IP3) and diacylglycerol (DG). As with the cyclic nucleotide system, the receptor-mediated stimulation of PLC is probably associated with a guanine nucleotide binding protein. Once formed, IP3 and DG serve as second messengers, with the former liberating intracellular stores of calcium ion and the latter stimulating the calcium-activated, phospholipid-dependent enzyme, protein kinase C (Ref. 14). Like the cAMP-dependent protein kinase, protein kinase C modifies cellular activity by catalysing the phosphorylation of various substrates.

It is conceivable that phosphatidylinositol turnover and adenylyl cyclase activation are interrelated. One way to address this issue is to examine the influence of protein kinase C on cAMP formation. This has been accomplished by studying the effect of phorbol esters on the cAMP-generating system. Phorbol esters mimic DG and thereby stimulate protein kinase C (Ref. 20). Thus, by exposing tissue slices to phorbol esters it is possible to assess whether activation of protein kinase C can modify the functioning of the receptor-coupled adenylyl cyclase system. Such experiments have revealed that phorbol esters, like o-adrenergic and GABA_B agonists, have no effect on cAMP formation themselves, but augment the cAMP response that occurs during exposure to agents that stimulate receptors that are positively coupled to adenylyl cyclase (Fig. 1). Such findings suggest that activation of protein kinase C, perhaps through stimulation of PLC, may play a role in mediating the augmenting response to GABA_B and o-adren-
ergic agonists. This conclusion is supported by the finding that o-adrenergic agonists both stimulate PI turnover and augment β-adrenergic receptor-mediated cAMP accumulation in rat pinealocytes and guinea-pig brain tissue. However, such a relationship is less obvious in rat brain slices where α-adrenergic receptors appear to participate in the cAMP augmenting response, whereas α-adrenergic receptors are primarily responsible for stimulating PI turnover. In addition, although GABA subtypes are capable of augmenting cAMP accumulation in rat brain slices, they apparently have no effect on PI turnover. Such data make it appear that PI turnover is not an obligatory step with regard to regulating neurotransmitter receptor-mediated cAMP accumulation in rat brain.

Possible relationship between phospholipase A2 and protein kinase C

The findings described above suggest that the augmentation of cAMP production can occur through more than one mechanism, one of which is associated with PLA2 and the other with PLC. On the other hand, some recent discoveries indicate a way in which PLA2 may be linked to protein kinase C without requiring the activation of PLC. Thus it has been reported that a variety of unsaturated fatty acids, including arachidonate, are capable of directly stimulating protein kinase C (Reis, 23, 24). If such an action occurs in vivo, then stimulation of PLA2, and the consequent liberation of arachidonic acid, could result in the activation of protein kinase C which, in turn, may mediate the cAMP augmenting phenomenon (Fig. 2). Accordingly, in this model the common denominator of the cAMP augmenting response is protein kinase C, with its activation resulting from either stimulation of PLC and the generation of DG and IP3, or stimulation of PLA2, and the release of arachidonic acid (Fig. 2).

There are several ways in which protein kinase C could regulate the neurotransmitter receptor-stimulated accumulation of cAMP. Two of these relate to effects on G protein (Fig. 2). Activation of protein kinase C could lead to the phosphorylation of Ga, increasing its activity or facilitating its interaction with the catalytic unit of adenylate cyclase. Alternatively, protein kinase C might catalyse the phosphorylation of Ga, diminishing its capacity to inhibit adenylate cyclase, thereby increasing the responsiveness of the cyclase system when it is activated through Ga. This latter hypothesis is supported by the finding that protein kinase C phosphorylates the α-subunit of Gα in platelet membranes, and by the finding that a 41 kDa protein, which may represent α-Ga, is phosphorylated by the membrane-bound protein kinase C. Whether the end result, the demonstration that activation of protein kinase C, through stimulation of PI turnover, or an increase in the availability of arachidonic acid, may be the critical step that subserves the o-adrenergic and GABAβ receptor regulation of cAMP responses in brain.

Biological significance

Much of the data to support the present hypothesis has, by necessity, been obtained from indirect experiments. However, it is not possible that similar conclusions have been reached using different approaches. For example, although methoxamine is known to be a nonselective ligand of PLA2, the results with this agent, taken together with those from the EGA and glucocorticoid studies, provide strong support for an involvement of PLA2 in the α-adrenergic and GABAβ receptor-mediated augmentation phenomenon. From a physiological and pharmacological standpoint, these findings may be of significance in explaining why neurotransmitter receptor sensitivity remains stable under normal conditions. That is, the o-adrenergic and GABAβ augmenting action could allow for a greater biochemical response to be achieved with a smaller quantity of neurotransmitter (Fig. 1). Since the rate and extent of desensitization is thought to be a function of receptor occupancy, enhancement of the response beyond the level of the recognition site might serve as a means for preventing receptor desensitization. Inasmuch as the pharmacological manipulation of this augmenting effect could lead to subtle alterations in neurotransmitter receptor function, these findings have important implications with respect to the treatment of a variety of psychiatric and neurological illnesses.

Acknowledgments

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Airway neuropeptides and asthma

Peter J. Barnes

Many neuropeptides have recently been identified in airways and have potent effects on airway caliber and secretions, raising the possibility that they may be involved in airway diseases such as asthma. Vasoactive intestinal peptide and peptide histidine methionine are potent bronchodilators and may be neurotransmitters of non-adrenergic inhibitory nerves in airways. In asthma, if these peptides are broken down more rapidly by enzymes from inflammatory cells, the effect might contribute to exaggerated bronchial responsiveness. Sensory neuropeptides, such as substance P, neurokinin A and calcitonin gene-related peptide might contribute to the pathogenesis of asthma if released from stimulated unmyelinated nerve endings by an axon reflex. Some of the recent studies on airway neuropeptides are reviewed here by Peter Barnes with particular emphasis on how neuropeptides might be implicated in asthma.

A large number of neuropeptides have been found in the gut, where they may have important roles in regulation of motility and secretion. Because airways are derived embryologically from foregut it is not surprising to find these same neuropeptides in the respiratory tract. While the precise physiological role of neuropeptides in airways remains uncertain, the potent effects of these peptides on various aspects of airway function suggest that they may be involved in controlling airway tone and secretions. It is possible that functional development of neurogenic nerves might be involved in airway disease, and particularly asthma.

Asthma, which is characterized by bronchial hyperresponsiveness, or excessive 'twitchiness' of the airways, was attributed to abnormal nervous mechanisms until the middle of the present century, when immunological and mediator theories of pathogenesis gained favour. The recent demonstration of an extensive network of potent neuropeptides in the airways has revived interest in possible neural abnormalities in asthma. Recent experimental and clinical studies suggest that bronchial hyperresponsiveness may be explained by an inflammatory response in the airway wall. Neural, and neuropeptide control mechanisms might be involved in mechanisms that contribute to the inflammatory response.

Non-adrenergic non-cholinergic (NANC) nerves

Neural control of airways is more complex than previously recognised. In addition to classical cholinergic and adrenergic pathways, neural mechanisms which are neither adrenergic nor cholinergic have been described, as in the gut. Originally it was thought that purines such as adenosine or ATP might be neurotransmitters of NANC nerves in airways, but there is little evidence to support this idea and it now seems more likely that, as in the gastrointestinal tract, neuropeptides may be involved. Both excitatory and inhibitory NANC mechanisms have been described in airways, but the physiological significance of these pathways will remain uncertain until specific blockers become available. In human airways, NANC inhibitory nerves provide the only inhibitory nervous pathway, since direct adrenergic innervation of airway smooth muscle is lacking.

Vasoactive intestinal peptide (VIP)

VIP, a 28 amino acid peptide originally discovered as a vasoactive substance in lung extracts, potentially causes airway smooth muscle contraction. VIP has been localized to nerves in human and animal lungs; in human airways VIP-immunoreactive nerve fibers are associated with airway smooth muscle in systemic and, possibly, in the airway wall.
MANY neurotransmitters influence neuronal function by directly affecting either of two important signal transduction mechanisms. Of these mechanisms, the most well-characterized is the receptor-coupled adenylate cyclase system. Stimulation of the cyclase enzyme enhances the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), while inhibition decreases cAMP production. The cyclic nucleotide then acts as a "second messenger" by stimulating a protein kinase that phosphorylates selected proteins.

The other important receptor-coupled effector system is phosphatidylinositol turnover. In response to receptor activation, phospholipase C degrades phosphatidylinositol 4,5-bisphosphate, a phospholipid, to dual second messengers. One messenger, inositol triphosphate, liberates calcium ions from internal stores; the other, diacylglycerol, stimulates protein kinase C that, like the cAMP-dependent enzyme, phosphorylates multiple protein substrates.

The ability of neurotransmitters to influence these processes has routinely been examined in brain slices, intact tissue preparations that closely approximate in vivo conditions. These slices are maintained in an aerated, physiological buffer and are pre-labelled with a substrate precursor such as ['H]-adenine, which is converted to ['H]-ATP, or ['H]-inositol, which is incorporated into phospholipid pools. Enzyme activity in response to receptor activation can then be assessed by measuring the levels of radiolabelled product that have accumulated.

**Mixed reception**

Given the importance of identifying the events which follow receptor activation, the brain slice preparation is a valuable tool. Recent studies have shown that, although the direct stimulation of second messenger formation by neurotransmitters is often sufficient to account for the biochemical effects they provoke, many neurotransmitter receptors are also subject to the regulatory influences of compound pools acting at distinct receptor sites. Such compounds are called "neuromodulators" since they indirectly modify the biochemical response resulting from activation of another receptor.

For example, brain slices exposed to noradrenaline which stimulates both α- and β-adrenergic receptors, show a greater accumulation of cyclic AMP than those treated with isoprenaline, a selective β-adrenergic agonist (Table 1). These results suggest that α-adrenergic receptors, like the β-adrenergic components, may be directly coupled to adenylate cyclase. But α-agonists such as 6-fluorono-adrenaline (6-FNA) do not of themselves stimulate cAMP accumulation (Table 1).

**Table 1 Neurmodulator effects on cAMP accumulation**

<table>
<thead>
<tr>
<th>cAMP accumulation (%) conversion</th>
<th>Basal</th>
<th>Isoprenaline</th>
</tr>
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<tbody>
<tr>
<td>No additions</td>
<td>0.06</td>
<td>0.38</td>
</tr>
<tr>
<td>6-FNA (10 μM)</td>
<td>0.07</td>
<td>0.77</td>
</tr>
<tr>
<td>Baciolten (50 μM)</td>
<td>0.11</td>
<td>1.14</td>
</tr>
<tr>
<td>Diacylglycerol (100 μM)</td>
<td>0.06</td>
<td>1.20</td>
</tr>
<tr>
<td>Noradrenaline (100 μM)</td>
<td>0.80</td>
<td>0.80</td>
</tr>
</tbody>
</table>

The influence of various agents on cAMP accumulation in rat brain cerebral cortical slices. Results are expressed as per cent conversion, which represents the percentage of total tritium present as ['H]-cyclic AMP. 6-FNA, 6-fluorono-adrenaline; baciolten, β-chlorophenyl γ-amino butyric acid; PDBu, phorbol 12, 13-dibutyrate. (Adapted from refs 3,7,9.)

When, however, rat brain slices are exposed to both 6-FNA and isoprenaline, the cAMP response equals that obtained when noradrenaline alone is used. These findings can be taken as evidence that α-adrenergic receptors are indirectly coupled to the adenylate cyclase system and that noradrenaline, through interaction with these sites, can function as a neuromodulator.

Similar results have recently emerged from studies using the agonist β-chlorophenyl γ-amino butyric acid (baciolten). Baciolten specifically recognizes a γ-amino butyric acid (GABA) receptor subtype which is referred to as the GABA_A site. Like 6-FNA, baciolten augments cAMP accumulation markedly in brain slices following exposure to a number of β-adrenergic receptor stimulants, while being ineffective alone in this regard (Table 1). The augmenting response elicited by both GABA_A and α-adrenergic agonists is independent of phosphodiesterase activity and totally dependent upon the presence of extracellular calcium, suggesting a common mechanism of action.

The ability to augment cyclic AMP formation is not limited to endogenous substances, for it can be elicited by phorbol esters, compounds that mimic the action of diacylglycerol and activate protein kinase C (Table 1). This finding is intriguing in that it suggests that activation of phospholipase C and the subsequent generation of diacylglycerol may be the mechanism responsible for the augmentation observed in response to GABA_A and α-adrenergic receptor activation. Such a mechanism would point to a positive interaction between two major signalling pathways.

**A cut above**

Brain slice preparations have been invaluable in elucidating a role for α-adrenergic and GABA_A receptors, because the augmentation phenomenon is not observed in cell-free preparations. The reason for this absence is not obvious, but may be related to the fact that direct receptor-mediated stimulation of adenylate cyclase is also difficult to detect in membrane preparations. Alternatively, a requirement for some soluble factor may preclude the detection of any augmentation in isolated plasma membranes. Since the augmentation response is a functional measure of receptor activity, this system can be used to screen for potential α-adrenergic and GABA_A receptor agonists and antagonists, which cannot be identified with receptor binding analysis. Brain slice preparations can also disclose the biochemical consequences of agents like phorbol esters that act at intracellular sites. And while the physiological relevance of receptor-mediated cAMP augmentation is still unknown, brain slice studies have hinted that the pharmacological manipulation of neuromodulator receptors might hold considerable therapeutic potential.

William Karbon is at the Department of Pharmacology, Yale University School of Medicine, PO Box 3333, New Haven, Connecticut 06510, USA. Salvatore Enna is research director at Novo Pharmaceutical Corp, 5210 Eastern Avenue, Baltimore, Maryland 21224, USA.

An Examination of the Involvement of Phospholipases A2 and C in the α-Adrenergic and γ-Aminobutyric Acid Receptor Modulation of Cyclic AMP Accumulation in Rat Brain Slices

R. S. Duman, E. W. Karbon, *C. Harrington, and S. J. Enna

Departments of Pharmacology, Neurobiology and Anatomy, and *Psychiatry, University of Texas Medical School, Houston, Texas, U.S.A.

Abstract: Experiments were undertaken to define the role of two calcium-associated enzyme systems in modulating transmitter-stimulated production of cyclic nucleotides in rat brain. Cyclic AMP (cAMP) accumulation was examined in cerebral cortical slices using a prelabeling technique. The enhancement of isoproterenol-stimulated cAMP production by α-adrenergic and γ-aminobutyric acid-B (GABA_B) agonists was reduced by exposing the tissue to EGTA, a chelator of divalent cations, or quinacrine, a nonselective inhibitor of phospholipase A2. Likewise, chronic (2 weeks) administration of corticosterone decreased the α-adrenergic and GABA_B receptor modulation of second messenger production. Neither cyclooxygenase nor lipooxygenase inhibitors selectively influenced the facilitating response of α-adrenergic and GABA_B agonists. Other experiments revealed that although norepinephrine and 6-fluoronorepinephrine stimulated inositol phosphate (IP) production in cerebral cortical slices with potencies equal to those displayed in the cyclic nucleotide assay, selective α-adrenergic agonists were less efficacious on IP formation and were without effect in the cAMP assay. Conversely, a selective α2-adrenergic receptor agonist facilitated the cAMP response to a β-adrenergic agonist without affecting IP formation. The rank orders of potency of a series of α-adrenergic antagonists suggest that IP accumulation is mediated solely by α2-adrenergic receptors, whereas the augmentation of cAMP accumulation is regulated by a mixed population of α-adrenergic sites. The results suggest that the α-adrenergic and GABA_B receptor-mediated enhancement of isoproterenol-stimulated cAMP formation appears to be more closely associated with phospholipase A2 than phospholipase C and may be mediated by arachidonate or some other fatty acid. Key Words: Phospholipase A2—Phospholipase C—Cyclic AMP accumulation—α-Adrenergic receptor—γ-Aminobutyric acid receptor—Rat brain. Duman R. S. et al. An examination of the involvement of phospholipases A2 and C in the α-adrenergic and γ-aminobutyric acid receptor modulation of cyclic AMP accumulation in rat brain slices. J. Neurochem. 47, 800–810 (1986).

One way in which brain neurotransmitters modify neuronal activity is by stimulating the production of cyclic AMP (cAMP), a second messenger that regulates intracellular kinase activity and protein phosphorylation (Bloom, 1975). However, not all brain receptors are capable of influencing cAMP production through a direct coupling to adenylate cyclase. For example, activation of α-adrenergic sites has no effect on cAMP formation but greatly enhances the response to stimulating agents (Perkins and Moore, 1973; Daly et al., 1980). This explains why the nonselective adrenoceptor agonist norepinephrine is a more efficacious activator of cAMP formation than the selective β-adrenergic agonist isoproterenol. Similarly, γ-aminobutyric acid (GABA) itself or GABA_B receptor agonists, such as baclofen, have little effect on basal cAMP levels in brain tissue. Instead, they enhance second messenger accumulation in response to a variety of agents, including isoproterenol.

Abbreviations used: ACTH, adrenocorticotropic hormone; cAMP, cyclic AMP; DG, diacylglycerol; GABA, γ-aminobutyric acid; IP, inositol phosphate; PLA2, phospholipase A2.
adrenosine, and vasoactive intestinal peptide (Karbon et al., 1984; Karbon and Enna, 1985). These data suggest that norepinephrine and GABA, acting through α-adrenergic and GABA receptors, respectively, serve a neuromodulatory as well as a neurotransmitter role in CNS function.

The components of receptor systems directly coupled to adenylyl cyclase have been defined to some extent (Gilman, 1984), but little is known about the mechanisms whereby neuromodulators regulate receptor responsiveness, although extra-cellular calcium appears to be required for the augmenting activity (Schwabe and Daly, 1977; Karbon and Enna, 1985). This suggests that the α-adrenergic and GABAergic enhancement of cAMP production may be mediated through enzyme systems associated with Ca²⁺, such as phospholipase A₂ (PLA₂) and phospholipase C. The former is a likely candidate because it has been suggested that pros-taglandins participate in the α-adrenergic receptor-mediated augmentation in cAMP production (Par- 
ington et al., 1980). For phospholipase C, α-adrenergic agonists activate this enzyme in brain tissue, catalyzing the degradation of polyphosphoinositides and the formation of inositol phosphates (IPs), which regulate the intracellular calcium level, and diacylglycerol (DG), which stimulates C kinase, a calcium-activated enzyme (Berridge and Irvine, 1984; Brown et al., 1984). Evidence that this system may contribute to the α-adrenergic receptor-mediated augmentation of cAMP production is provided by reports that phorbol esters, substances known to stimulate C kinase directly, influence CAMP production in a manner similar to the neuromodulators (Bell et al., 1985; Hollingsworth et al., 1985; Karbon et al., 1985; Sugden et al., 1985). Because both phorbol esters and α-adrenergic agonists stimulate C kinase, although by different mechanisms, it is possible this enzyme may be an important link with regard to the modulation of cAMP production in brain.

The present study was undertaken to examine these issues by exploring the relationships among PLA₂, IP formation, and the augmentation of transmitter receptor-stimulated cAMP production in rat brain cerebral cortex. The results suggest that PLA₂ may be an important component of the neuromodulatory response to α-adrenergic and GABA receptors, whereas a role for IP remains questionable.

MATERIALS AND METHODS

Male Sprague-Dawley rats (body weight 125-150 g) were used for all experiments. The animals were housed under a 12-h light-dark cycle with food and water ad libitum. For some studies, Acthar gel (150 IU/kg s.c.) or corticosterone (15 mg/kg s.c.) suspended in corn oil was administered once a day for 14 consecutive days. The rats were killed by decapitation 18 h after the last injection.

The accumulation of cAMP was measured in brain slices using the prelabeling technique of Shimizu et al. (1969). In brief, the cerebral cortex was removed and minced into slices 350 μm thick using a McIlwain tissue chopper. The slices were suspended in an oxygenated (95% O₂, 5% CO₂) Krebs-Ringer-bicarbonate buffer containing 120 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 1.0 mM KH₂PO₄, 20 mM NaHCO₃, and 11.1 mM glucose. After decantation, fresh buffer was added, and the slices were incubated for 15 min at 37°C. The slices were then placed into buffer containing [³²P]adenine (29 Ci/mmol, 4.0 μCi/ml) and incubated for 60 min at 37°C. The medium was decanted, the slices were washed twice with buffer, and portions (~25 mg wet weight) were incubated in a 500-μl volume for 5 min at 37°C. For some experiments, the slices were subsequently exposed to various drugs for up to 30 min before addition of norepinephrine, isoproterenol, 6-fluoronorepinephrine, or baclofen. Incubation was continued for another 15 min before the reaction was terminated by addition of 10% trichloroacetic acid (550 μl). The samples were then homogenized and centrifuged at 10,000 × g for 10 min. Total radioactivity was monitored in a 50-μl portion of the acid supernatant, and the cAMP content was analyzed by the double-column method of Salomon et al. (1974) using [³²P]cAMP to measure recovery. The results are expressed as the percentage of total tritium present as [³²P]cAMP (percentage conversion).

The method of Brown et al. (1984) was used to analyze IP accumulation in brain tissue. Cerebral cortical slices were prepared as above and incubated for 45 min at 37°C with a change of buffer every 5 min. The slices were incubated an additional 15 min at 37°C in the presence of 5 mM LiCl, after which, [³²P]inositol (40 Ci/mmol, 1.0 μCi/ml) was added and the samples were incubated for another 30 min. Following this incubation, the tissue was exposed to the receptor agonists for 45 min. In some experiments, receptor antagonists were added 5-10 min before the receptor agonists. The reaction was terminated by addition of 940 μl of a chloroform methanol (1:2 vol/vol) mixture, and after addition of 320 μl of chloroform and 320 μl of water, the samples were centrifuged at 1,000 g. [³²P]IP was extracted from a 750-μl portion of the aqueous phase by ion exchange chromatography ( Dowex 200-400 mesh, chloride form). The resin was washed four times with 3.0 ml of water, and [³²P]IP was removed with 1.0 M ammonium formate and quantified by liquid scintillation spectrometry. [³²P]IP accumulation is expressed as a percentage over the basal rate.

(-)-Isoproterenol HCl, (-)-norepinephrine bitartrate, (l)-phenylephrine HCl, quinacrine HCl, EGTA, indomethacin, acetylsalicylic acid, acetaminophen, flufenamic acid, ibuprofen, nordihydroguaiaretic acid, corticosterone, yohimbine, and cAMP were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [³²P]Adenine was purchased from ICN (Irvine, CA, U.S.A.). [³²P]inositol from New England Nuclear (Boston, MA, U.S.A.). Acthar gel from Armour Pharmaceuticals (Phoenix, AZ, U.S.A.), and WB 4101 [2:6-dimethoxyxphenoxymethylaminomethenol-1,4-doxan] from Amersham Corp. (Chicago, IL, U.S.A.). [³²P]Fluoro- norpinephrine was kindly donated by Dr. K. L. Kirk of the National Institutes of Health (Bethesda, MD, U.S.A.), cizazoline by Dr. S. Langer of L.E.R.S.-Syn- thelabo (Paris, France), baclofen by Ciba Geigy (Basel, Switzerland).

RESULTS

Norepinephrine-stimulated cAMP accumulation was some two to three times greater than that found with the \( \beta \)-adrenergic agonist isoproterenol (Fig. 1). Likewise, the cAMP response to the isoproterenol/baclofen combination was much greater (fourfold) than to isoproterenol alone (Fig. 1). Neither baclofen alone nor \( \alpha \)-adrenergic agonists have any significant effect on cAMP accumulation under these conditions (Daly et al. 1980; Karbon et al., 1984). The cAMP accumulation in the presence of GABA or \( \alpha \)-adrenergic agonists exceeding that observed with isoproterenol alone is defined as the augmenting or facilitating response.

Exposure to EGTA completely eliminated the augmenting response but had no effect on the \( \beta \)-adrenergic agonist alone (Fig. 1). The concentration of EGTA causing half-maximal inhibition (IC\( _{50} \)) was \( \sim 1.0 \) mM for both norepinephrine and the isoproterenol/baclofen combination.

Like EGTA, quinacrine inhibited the norepinephrine and isoproterenol-baclofen response in a concentration-dependent manner without affecting the response to isoproterenol alone (Fig. 2). The IC\( _{50} \) for quinacrine was \( \sim 110 \mu M \) in both cases. Although quinacrine completely eliminated the augmenting response to norepinephrine, 20% of the baclofen-induced augmentation was unaffected by quinacrine (Fig. 2). With regard to IP formation, it was impossible to determine whether quinacrine affected norepinephrine-stimulated production of IP, because it increased the basal levels of IP (data not shown).

Chronic (2 weeks) administration of corticosterone or adrenocorticotrophic hormone (ACTH) significantly reduced norepinephrine-stimulated cAMP accumulation in rat brain cerebral cortical slices without affecting the response to isoproterenol (Table 1). Furthermore, both treatments reduced the augmenting observed with the GABA\( _B \) agonist baclofen or the \( \alpha \)-adrenergic agonist 6-fluoronorepinephrine. For norepinephrine and the isoproterenol/6-fluoronorepinephrine combination, the corticosterone and ACTH treatments decreased the augmenting response by almost 50%. The facilitating response observed in the presence of isoproterenol/baclofen was decreased \( \sim 30\% \) by these treatments (Table 1).

The influence of chronic (16 days) administration of ACTH on IP accumulation was also examined. ACTH treatment did not alter the amount of nor-
### TABLE 1. Effect of chronically administered ACTH or corticosterone on receptor-stimulated cAMP and IP accumulation in rat brain cerebral cortical slices

<table>
<thead>
<tr>
<th>Accumulation, receptor agonist</th>
<th>Treatment</th>
<th>Vehicle</th>
<th>ACTH</th>
<th>Corticosterone</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]cAMP (µM conversion)</td>
<td>Basal</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.02</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>ISO</td>
<td>0.37 ± 0.04</td>
<td>0.33 ± 0.03</td>
<td>0.33 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>NE</td>
<td>0.97 ± 0.06</td>
<td>0.62 ± 0.06*</td>
<td>0.66 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>ISO+NE</td>
<td>0.91 ± 0.06</td>
<td>0.54 ± 0.03*</td>
<td>0.96 ± 0.06*</td>
</tr>
<tr>
<td></td>
<td>ISO-baclofen</td>
<td>1.05 ± 0.09</td>
<td>0.86 ± 0.04*</td>
<td>0.81 ± 0.09*</td>
</tr>
<tr>
<td>[3H]IP (cpm)</td>
<td>Basal</td>
<td>806 ± 95</td>
<td>1,356 ± 113*</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NE</td>
<td>2,562 ± 294</td>
<td>2,679 ± 121</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Baclofen</td>
<td>6,633 ± 507</td>
<td>6,541 ± 321</td>
<td>—</td>
</tr>
</tbody>
</table>

Rats were given ACTH (1 mg kg⁻¹) or corticosterone (15 mg kg⁻¹) once a day for 14 consecutive days and then killed 12 h after the last injection. Isoproterenol (ISO), ISN or norepinephrine (NE, 100 µM)-stimulated cAMP accumulation were measured in cerebral cortical slices using a prelabeling technique. In some cases, the cAMP response was measured in the presence of ibuprofen (ISO), ISN or norepinephrine (NE, 100 µM) or baclofen (ISO). Data are mean ± SEM values from four to six separate experiments, each of which was analyzed in duplicate.

* p < 0.05 compared with the corresponding control (Student's t-test)

### FIG. 2. Quinacrine inhibition of norepinephrine-stimulated and baclofen-induced enhancement of isoproterenol-stimulated cAMP accumulation. Slices of rat brain cerebral cortex were prelabeled with [3H]adenine and then incubated with various concentrations of quinacrine for 15 min. Following this, they were exposed to either norepinephrine (100 µM) or isoproterenol (100 µM) plus baclofen (50 µM) for 10 min. In both cases, the IC₅₀ for quinacrine was ~110 µM. The response to a saturating concentration (20 µM) of isoproterenol alone was 0.49 ± 0.02 (stippled line). Data are mean ± SEM (bars) values from three to five separate experiments, each of which was analyzed in duplicate.

### TABLE 2. Effect of cyclooxygenase and lipoygenase inhibitors on receptor-stimulated cAMP accumulation in rat brain slices

<table>
<thead>
<tr>
<th>Enzyme inhibitor</th>
<th>ISO</th>
<th>NE</th>
<th>ISO + Baclofen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.22 ± 0.03</td>
<td>1.19 ± 0.10</td>
<td>1.26 ± 0.09</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>0.30 ± 0.02</td>
<td>1.06 ± 0.12</td>
<td>1.10 ± 0.15</td>
</tr>
<tr>
<td>Acetylsalicylic acid</td>
<td>0.33 ± 0.02</td>
<td>1.16 ± 0.08</td>
<td>1.22 ± 0.13</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>0.38 ± 0.03</td>
<td>1.06 ± 0.10</td>
<td>1.20 ± 0.18</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>0.30 ± 0.04</td>
<td>1.00 ± 0.06</td>
<td>1.07 ± 0.09</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>0.16 ± 0.02*</td>
<td>0.14 ± 0.02*</td>
<td>0.14 ± 0.02*</td>
</tr>
<tr>
<td>Nordihydroguaiaretic acid</td>
<td>0.21 ± 0.03*</td>
<td>0.69 ± 0.06*</td>
<td>0.63 ± 0.06*</td>
</tr>
<tr>
<td>Eicosatetraynoic acid</td>
<td>0.12 ± 0.02*</td>
<td>0.15 ± 0.02*</td>
<td>0.23 ± 0.04*</td>
</tr>
</tbody>
</table>

Slices of rat brain cerebral cortex were prepared and prelabeled with [3H]adenine as described in Materials and Methods. The slices were incubated for 30 min with one of various cyclooxygenase and lipoygenase inhibitors (100 µM), after which they were incubated for 15 min with 20 µM isoproterenol (ISO). 100 µM norepinephrine (NE) or the combination of ISO and baclofen (ISO). Data are mean ± SEM values from three to five separate experiments, each of which was performed in duplicate.

* p < 0.05 compared with the corresponding control (Student's t-test)
FIG. 3. Adrenergic agonist stimulation of IP and cAMP accumulation in rat brain slices. Slices of cerebral cortex were pre-labeled with [3H]inositol (upper panel) or [3H]adenine (lower panel) for determination of IP or cAMP formation, respectively. The slices were exposed to one of various concentrations of the agonists, and the half-maximal effective concentration (EC50) for each agent was determined by log-probit analysis. Norepinephrine, 6-fluoronorepinephrine, and cirazoline-stimulated IP production yielded EC50 values of 6.8 ± 1.9, 9.3 ± 1.0, and 0.18 ± 0.1 μM, respectively. The EC50 values for norepinephrine and 6-fluoronorepinephrine to enhance isoproterenol-stimulated cAMP production were 7.5 ± 0.8 and 6.1 ± 0.4 μM, respectively. The cAMP assay was conducted in the presence of a saturating concentration (20 μM) of isoproterenol to reveal the α-adrenergic component of norepinephrine. The response to a saturating concentration of isoproterenol alone was 0.24 ± 0.03 (stippled line). Data are mean ± SEM (bars) values from three to five separate experiments, each of which was performed in duplicate.

Neither cyclooxygenase nor lipoxygenase inhibitors selectively reduced the augmenting response (Table 1). Exposure to a high concentration (100 μM) of indomethacin, acetylsalicylic acid, acetaminophen, or ibuprofen had no significant effect under any of the conditions studied. Although flufenamic, nordihydroguaiaretic, and eicosatetraynoic acids did reduce cAMP accumulation, they inhibited the response to isoproterenol alone, suggesting that they do not selectively modify the augmentation.

Norepinephrine stimulates IP and cAMP accumulation in rat brain slices in a concentration-dependent manner (Fig. 3). The α-adrenergic component of the norepinephrine-stimulated cAMP response was analyzed by conducting these experiments in the presence of a saturating concentration of isoproterenol (20 μM). The concentration of norepinephrine necessary to elicit a half-maximal response (EC50) was ~7 μM in both systems. Likewise, 6-fluoronorepinephrine augmented cAMP production and stimulated IP formation with equal potency (EC50 ~10 μM). However, although the maximal responses to norepinephrine and 6-fluoronorepinephrine are the same in the cAMP assay, the fluorinated analog was slightly less efficacious with respect to IP formation (Fig. 3). Cirazoline, a selective α1-adrenergic receptor agonist (van Meel et al., 1981; Cavero et al., 1982), was less efficacious than norepinephrine and 6-fluoronorepinephrine as an activator of IP formation but more potent than either of these agents (Fig. 3). In contrast, cirazoline was inactive as a stimulator or enhancer of cAMP production at concentrations up to 100 μM (Table 3). Like cirazoline, the selective α1-adrenergic receptor agonist phenylephrine (Wilkberg, 1977; Ruffolo, 1984) stimulated IP accumulation and was less efficacious than norepinephrine in
TABLE 3. Comparison of a-adrenergic receptor agonists as stimulators of IP and cAMP accumulation in rat brain cerebral cortical slices

<table>
<thead>
<tr>
<th>a-Adrenergic agonist</th>
<th>Maximum response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(PHIP accumulation</td>
</tr>
<tr>
<td>Phenytoin (100 µM)</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Phenylephrine (300 µM)</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>UK-14,304 (100 µM)</td>
<td>0.06 ± 0.01</td>
</tr>
</tbody>
</table>

Rat brain cerebral cortical slices were prepared and prelabeled with [3H]inositol or [3H]adenine as described in Materials and Methods. To study IP accumulation, the slices were incubated with norepinephrine (NE) or one of the other adrenergic agonists at the concentrations indicated. The data for IP accumulation are expressed as percentage stimulation over basal activity. For cAMP analysis, the slices were exposed to the adrenergic agonists in the absence (basal) or presence of isoproterenol (ISO; 20 µM). Data are mean ± SEM values from three to five separate experiments, each of which was performed in triplicate.

This regard (Table 3). Furthermore, phenylephrine had no effect on cAMP accumulation alone or in combination with isoproterenol.

Unlike the a1-adrenergic agonists, the selective a1-adrenergic receptor stimulant UK-14,304 (Cambridge, 1981; van Meel et al., 1981; Ruffolo, 1984) had no effect on IP production but did augmen isoproterenol-stimulated cAMP accumulation (Table 3). Simultaneous exposure to UK-14,304 and phenylephrine had no effect on cAMP production in the absence of isoproterenol but did facilitate the β-adrenergic receptor response to the same extent as that observed with UK-14,304 alone (Table 3).

Both cirazoline and phenylephrine caused a concentration-dependent inhibition of norepinephrine-stimulated IP production, with the former inhibiting 52% and the latter 24% at maximally effective concentrations (Fig. 4). Moreover, both agents completely eliminated the a-adrenergic component of the cAMP response to norepinephrine, with cirazoline being more potent than phenylephrine in this regard (Fig. 4).

The selective a2-adrenergic receptor antagonists prazosin, YM-12617, and WB 4101 (Cambridge et al., 1977; Bjuland and U’Prichard, 1983; Honda et al., 1985) completely inhibited norepinephrine-stimulated IP formation in the low nanomolar range, whereas the selective a2-adrenergic receptor antagonists WY 26392, yohimbine, and idazoxan (Chapelo et al., 1981; Puciorek and Shepperson, 1985) were much weaker, having IC50 values in the low micromolar range (Table 4 and Fig. 5). The rank order of potencies for inhibition of IP formation was YM-12617 > prazosin > WB 4101 > YW 26392 > idazoxan. With respect to cAMP production, prazosin and YM-12617 blocked the augmenting response with IC50 values in the nanomolar range, whereas WB 4101 was much weaker (Table 4). In addition, the inhibition curves for YM-12617 and WB 4101 were not monophasic, and prazosin blocked only 50% of the augmenting response (Fig. 5). The a2-adrenergic antagonists were weaker than YM-12617 and prazosin as inhibitors of cAMP augmentation, although the rank order of potencies differed for the group as compared with IP formation: YM-12617 > prazosin > idazoxan > WY 26392 > yohimbine > WB 4101 (Table 4 and Fig. 5).

DISCUSSION

Previous reports have suggested that a-adrenergic and GABAergic augmentation of brain cAMP production is a calcium-dependent process (Schwabe and Daly, 1977; Karbon and Enna, 1985) and that the a-adrenergic enhancement may be related to the production of prostaglandins (Partridge et al., 1980). These findings suggest that the a-adrenergic and GABAergic augmentation of cAMP production may be through activation of PLA2, a calcium-dependent enzyme that liberates the prostaglandin precursor arachidonic acid. Alternatively, the augmenting response to a-adrenergic, but not GABAergic, agonists may involve another calcium-activated enzyme, protein kinase C. Thus, unlike GABAergic agonists (Brown et al., 1984), a-adrenergic agonists are known to stimulate phospholipase C, catalyzing the breakdown of polyphosphoinositides with the subsequent formation of IPs and DG (Berridge and Irvine, 1984). IP, in turn, liberates intracellular stores of bound calcium, and DG stimulates C kinase. Although the intracellular concentration of calcium is normally sufficient for activating C kinase in the presence of DG, it is conceivable that an influx of extracellular calcium could influence enzyme activity as well. Moreover, it has recently been reported that phorbol esters, which directly stimulate C kinase, facilitate the cAMP response in brain tissue in a manner similar to that found for a-adrenergic and GABAergic agonists (Hollingsworth et al., 1985; Karbon et al., 1985). In addition, agents that stimulate phospholipid turnover have been shown to enhance receptor-mediated cAMP accumulation in guinea pig brain (Hollingsworth and Daly, 1985). The results of the present study confirm the importance of Ca2+ in the augmenting response and provide new data indicating that phospholipid metabolism may play a crucial role in modulating neurotransmitter receptor responses in brain.

As reported previously (Schwabe and Daly, 1977; Karbon and Enna, 1985), the present results indicate that exposure of rat brain slices to EGTA-com-
It:0
CIRAZOLINE
*

FIG. 4. Inhibition of norepinephrine-stimulated IP or cAMP accumulation by phenylephrine or cirazoline. Slices of cerebral cortex were prelabeled with either [3H]inositol (upper panel) or [3H]adenine (lower panel) for the determination of IP or cAMP production, respectively. Various concentrations of phenylephrine or cirazoline were added to the incubation medium 5-10 min before addition of norepinephrine (100 μM). The response to a saturating concentration (20 μM) of isoproterenol alone was 0.28 ± 0.01 (stippled line). Data are mean ± SEM (bars) values from three to five separate experiments, each of which was performed in duplicate.

**TABLE 4. Inhibition of norepinephrine-stimulated IP and cAMP accumulation in rat brain cerebral cortical slices by α-adrenergic receptor antagonists**

<table>
<thead>
<tr>
<th>α-Adrenergic antagonist</th>
<th>IC_{50} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[3H]IP accumulation</td>
</tr>
<tr>
<td>YM-22671</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Prazosin</td>
<td>26 ± 6</td>
</tr>
<tr>
<td>WB 4101</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>Yohimbine</td>
<td>8000 ± 1000</td>
</tr>
<tr>
<td>WY 26,3621</td>
<td>12,000 ± 2000</td>
</tr>
<tr>
<td>Idazoxan</td>
<td>18,000 ± 4000</td>
</tr>
</tbody>
</table>

Rat brain cerebral cortical slices were prelabeled with [3H]inositol or [3H]adenine as described in Materials and Methods. Various concentrations of each antagonist were added 5-10 min before the tissue was exposed to norepinephrine (100 μM). Concentrations causing half-maximal inhibition (IC_{50}) were determined by log-probit analysis. Data are mean ± SEM values from three to five separate experiments, each of which was performed in duplicate.

Completely eliminates the α-adrenergic and GABA_{B} receptor-mediated augmentation of receptor-stimulated cAMP production. The α-adrenergic and GABAergic systems appeared equally sensitive to Ca^{2+}, with the IC_{50} concentration for EGTA being identical in both cases. This suggests that α-adrenergic and GABAergic receptor activation allows calcium to enter the cell and mediate the augmenting response.

Given the previous suggestion that prostaglandins may facilitate the cAMP response to neurotransmitters (Partington et al., 1980), it seemed possible that calcium was necessary to activate PLA_{2} to catalyze the production of arachidonic acid. Quinacrine, a nonselective inhibitor of PLA_{2} (Billah and Lapetina, 1982; Snider et al., 1984), reduced the augmenting response observed with norepinephrine and baclofen. Whereas quinacrine inhibited norepinephrine-stimulated cAMP produc-
FIG. 5. Inhibition of norepinephrine-stimulated IP or cAMP accumulation by prazosin, YM-12,617, WB 4101, and yohimbin. Slices of cerebral cortex were prelabeled with either [3H]inositol (upper panel) or [3H]adenine (lower panel) for the determination of IP or cAMP production, respectively. Various concentrations of antagonists were added to the incubation medium 5–10 min before addition of norepinephrine (100 μM). The response to a saturating concentration (20 μM) of isoproterenol alone was 0.35 ± 0.05 (stippled line). Data are mean ± SEM (bars) values from three to five separate experiments, each of which was performed in duplicate.

Administration reduces the α-adrenergic component of norepinephrine-stimulated cAMP accumulation in rat brain (Duman et al., 1985). A mechanism for this action may be that the peptide stimulates the release of adrenal corticosteroids, which, in turn, promote the production of a protein (e.g., macrocortin) that inhibits PLA₂ (Blackwell et al., 1980). Because corticosteroid administration is known to decrease PLA₂ activity in a variety of tissues (Lewis, 1984), treatment with this hormone might attenuate the augmenting response to α-adrenergic and GABA₉ receptor agonists. The results indicated that chronic (2 weeks) administration of either ACTH or corticosterone decreased the cAMP response to norepinephrine and to the isoproterenol + fluoromethoxiphenephrine combinations without affecting the β-adrenergic receptor response. This finding is consistent with the notion that activation of PLA₂ is a consequence of α-adrenergic and GABA₉ receptor stimulation.
In contrast to cAMP accumulation, ACTH treatment had no effect on norepinephrine- or carbachol-stimulated IP formation, suggesting that the hormone-induced reduction in norepinephrine-stimulated cAMP accumulation is independent of catecholamine-stimulated IP production. However, ACTH administration increased the basal levels of IP, suggesting that the hormone treatment may influence IP production in some way.

The arachidonic acid formed by the action of PLA₂ is metabolized to a variety of products, one or more of which may mediate the augmenting response. Two enzymes involved in the metabolism of arachidonate are lipoxigenase and cyclooxygenase (Samuelsson, 1972; Hamberg and Samuelsson, 1974). However, inhibitors of these enzymes were incapable of selectively reducing the cAMP accumulation augmenting response to either α-adrenergic or GABA₉ receptor stimulation. This contrasts with an earlier report suggesting that inhibition of cyclooxygenase modified the α-adrenergic receptor-mediated facilitation of cAMP accumulation in rat brain slices (Partington et al., 1980). Nevertheless, the present results suggest that arachidonic acid, or some other product of phospholipid metabolism, may influence neurotransmitter receptor-coupled adenylate cyclase systems in brain.

Because the data implicating PLA₂ in the augmenting response were obtained indirectly, attempts were made to demonstrate conclusively that norepinephrine and baclofen stimulate PLA₂ activity or arachidonic acid production in brain. However, efforts to prelabel brain phospholipids with [³H]arachidonate were unsuccessful, apparently because an insufficient amount of the labeled substance was incorporated into the phospholipid pool. Likewise, it was not possible to detect a baclofenor norepinephrine-induced change in PLA₂ activity by measuring the formation of lysophosphatidylcholine after prelabeling the tissue with [³H]cholesterol. This failure may be due to the fact that α-adrenergic and GABA₉ agonists stimulate only a limited number of brain cells so that the amount of liberated [³H]lysophosphatidylcholine is too small to detect, given the total amount of tissue radioactivity. Thus, the hypothesis that the α-adrenergic and GABAergic regulation of cAMP production in brain is associated with the production of fatty acids must be considered tentative until methods capable of measuring brain PLA₂ activity are developed, or until selective antagonists of PLA₂ are found.

To examine the possible relationship between α-adrenergic stimulation of phosphatidylinositol turnover and the cAMP-augmenting response, a series of experiments were conducted to compare the pharmacological selectivity of the two systems. The results indicated that the potencies of norepinephrine, a nonselective adrenergic agonist, and 6-

fluoronorepinephrine, a more selective α-adrenergic agonist (Dalrymple et al., 1980), to stimulate IP formation and augment cAMP accumulation were the same, suggesting a possible relationship between the two actions. However, cirazoline and phenylephrine, selective α-adrenergic receptor agonists (van Meel et al., 1981; Cavero et al., 1982), were more potent, although less efficacious, than either norepinephrine or 6-fluoronorepinephrine in stimulating IP production but were incapable of augmenting the cAMP response to isoproterenol. A possible explanation for this finding is that cirazoline and phenylephrine are incapable of stimulating phosphatidylinositol turnover sufficiently to augment the cAMP response. This seems unlikely, because 6-fluoronorepinephrine facilitated isoproterenol-stimulated cAMP production at a concentration that enhanced phosphatidylinositol turnover to the same extent as the more selective α-adrenergic agonists. Thus, assuming that phenylephrine and cirazoline increase IP production by acting at α-adrenergic receptors, the results indicate that α-adrenergic enhancement of cAMP is not mediated by IP formation.

In contrast, the selective α₁-adrenergic agonist UK-14,304 (Cambridge, 1981; van Meel et al., 1981; Ruffolo, 1984) was without effect on IP formation but facilitated isoproterenol-stimulated cAMP accumulation. The selective β-agonist clonidine (Starke et al., 1974) is inactive with respect to IP accumulation (Minneman and Johnson, 1984) and does not enhance isoproterenol-stimulated cAMP accumulation at saturating concentrations of the β-agonist (Skolnick and Daly, 1975; Sawaya et al., 1977; Vetulani et al., 1977; Schultz and Kleefeld, 1979; Pilc and Enna, 1986). The ineffectiveness of clonidine on the cAMP system has been attributed to its partial agonist properties at α₁-adrenergic receptors (Pilc and Enna, 1986). The fact that combining α₁- and α₂-adrenergic agonists had no greater effect on cAMP production than the α₁-adrenergic drug alone suggests further that IP production does not contribute to the cAMP accumulation augmenting response in rat brain.

Inasmuch as cirazoline and phenylephrine were substantially less efficacious than norepinephrine in the IP assay, it was possible these substances may be partial agonists for the receptor mediating this response. Indeed, experiments revealed that both reduced the IP response to norepinephrine, supporting a partial agonist action. Furthermore, both agents virtually abolised the augmenting component of the norepinephrine response in the cAMP assay, indicating little agonist activity at this receptor. These findings agree with an earlier report suggesting that the α₁-adrenergic receptor-mediated augmentation of cAMP production in brain is inhibited by α₁-adrenergic agonists (Mobley and Suber, 1978).
The pharmacological characteristics of the α-adrenergic receptors associated with IP production and the augmentation of cAMP accumulation were defined further by examining the effect of a number of α-adrenergic receptor antagonists. Norepinephrine-stimulated IP accumulation was antagonized by this group of drugs with a rank order of potency characteristic of an α₁-adrenergic receptor subtype. Thus, the more selective α₁-agonist prazosin, YM-12617, and WB 4101 (Cambridge et al., 1977; Bvlund and U’Prichard, 1983; Honda et al., 1985) were all more potent than the α₂-agonists yohimbine, YM-12617, and idazoxan (Chapelo et al., 1981; Pautrek and Shepperson, 1985). These findings are in agreement with previous reports suggesting that norepinephrine-stimulated IP production in brain is mediated by α₁-adrenergic receptors (Brown et al., 1984; Minneman and Johnson, 1984).

With regard to α-adrenergic augmentation of cAMP accumulation, the α₁-adrenergic receptor antagonists prazosin and YM-12617 were once again most potent, but the selective α₁-adrenergic receptor antagonists were more active than WB 4101, another α₁-adrenergic receptor antagonist. It was also noteworthy that prazosin, unlike yohimbine, does not completely inhibit the α₁-adrenergic augmentation of cAMP accumulation (Duman et al., 1985) and that neither YM-12617 nor WB 4101 inhibited this response in a monophasic manner, suggesting a mixture of α₁-adrenergic receptor subtypes (Pile and Enna, 1986). These results suggest that α₁-adrenergic receptor augmentation of cAMP accumulation may be coupled to several types of α₁-adrenergic receptors, whereas IP formation is associated only with the α₁-receptor subtype. Because α₁-adrenergic agonists stimulate IP formation but do not enhance isoproterenol-stimulated cAMP production, it appears that phosphatidylinositol turnover may not contribute to the cAMP response. However, this conclusion must be tempered by the realization that the heterogeneous nature of the brain slice preparation makes it difficult to disprove absolutely an association between IP turnover and cAMP production.

Even though the role of IP may be doubtful, the possibility remains that C kinase is a participant in this response. It has recently been reported that oleic and arachidonic acids can stimulate C kinase (McPhail et al., 1984; Murakami and Routenberg, 1985), making it conceivable that α₁-adrenergic or GABAergic receptor stimulation activates this enzyme by stimulating the production of a fatty acid. As has been shown for platelet membranes (Katada et al., 1985), C kinase may then phosphorylate one or more of the proteins associated with the receptor-coupled adenylate cyclase system. Such a modification of brain tissue could be responsible for the augmentation in cAMP accumulation noted in the present study. Although highly speculative, this model fits the existing data and provides a plausible mechanism for explaining the neuromodulatory action of GABAergic and α₁-adrenergic receptors in brain.

Acknowledgment: This work was supported in part by a U.S. Air Force contract, by grant BNS-82-15427 from the National Science Foundation, and by a grant from Bristol-Myers, Inc. S.J.E. is the recipient of U.S. Public Health Service Research Scientist Development Award MH-00501. We thank Mrs. Constance Chiappetta for her excellent technical assistance and Dr. K. Kirk for the supply of 6-fluoronephrine.

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Phorbol Esters Enhance Neurotransmitter-Stimulated Cyclic AMP Production in Rat Brain Slices

E. W. Karbon, S. Shenolikar, and S. J. Enna

Departments of Pharmacology and of Neurobiology and Anatomy, University of Texas Medical School, Houston, Texas, U.S.A.

Abstract: The effect of phorbol esters on cyclic AMP production in rat CNS tissue was examined. Using a prelabeling technique for measuring cyclic AMP accumulation in brain slices, it was found that phorbol 12-myristate, 13-acetate (PMA) enhanced the cyclic AMP response to forskolin and a variety of neurotransmitter receptor stimulants while having no effect on second messenger accumulation itself. A short (15-min) preincubation period with PMA was required to obtain maximal enhancement, whereas the augmentation was lessened by prolonged exposure (3 h) to the phorbol. The response to PMA was concentration dependent (EC_{50} = 1 \mu M) and regionally selective, being most apparent in forebrain, and was not influenced by removal of extracellular calcium or by inhibition of phosphodiesterase or phospholipase A_{2}. Only those phorbols known to stimulate protein kinase C augmented the accumulation of cyclic AMP. Moreover, the membrane substrates phosphorylated by endogenous C kinase and by a partially purified preparation of this enzyme were similar. The results suggest that phorbol esters, by activating protein kinase C, modify the cyclic AMP response to brain neurotransmitter receptor stimulation in brain by influencing a component of the adenylyl cyclase system beyond the transmitter recognition site. Key Words: Cyclic AMP—Phorbol 12-myristate, 13-acetate—Phorbol esters—Brain—Neurotransmitter stimulation—Protein kinase C. Karbon E. W. et al. Phorbol esters enhance neurotransmitter-stimulated cyclic AMP production in rat brain slices. J. Neurochem. 47, 1566-1575 (1986).

Numerous factors regulate the rate and extent of neurotransmitter- and hormone-stimulated cyclic AMP production in biological tissue (Gilman, 1984; Lefkowitz et al., 1984). These include a receptor recognition site, stimulatory and inhibitory guanine nucleotide binding proteins (N_{s} and N_{i}, respectively), the catalytic unit of adenylate cyclase, and phosphodiesterases. Some neurotransmitter receptors are directly coupled to the cyclic AMP-generating system in the mammalian brain, whereas others are indirectly linked to second messenger production (Daly et al., 1981; Drummond, 1983; Karbon et al., 1984; Magistretti and Schorderet, 1985). Examples of the latter include brain \(\alpha\)-adrenergic and \(\gamma\)-aminobutyric acid B (GABA_{B}) receptors. activation of which fails to stimulate production of cyclic AMP but amplifies the response to other receptor agonists. This action of GABA and \(\alpha\)-adrenergic agonists is dependent on the presence of extracellular calcium, a result suggesting this ion may be an important mediator of the augmenting response (Schwabe and Daly, 1977; Karbon and Enna, 1985; Duman et al., 1986).

Recently, it has been demonstrated that many hormones and neurotransmitters stimulate the metabolism of polyphosphoinositides, generating the production of at least two intracellular messengers, inositol triphosphate (IP_{3}) and diacylglycerol (DAG) (Berridge, 1984; Berridge and Irvine, 1984; Brown et al., 1984; Janowsky et al., 1984; Nishizuka, 1984). IP_{3} is reported to liberate calcium from membrane-bound stores, whereas DAG stimulates a calcium-activated, phospholipid-dependent enzyme, protein kinase C, which, along with calcium, mediates a variety of cellular responses (Berridge, 1984). Studies aimed at exami-
PHORBOL ESTERS AND BRAIN CYCLIC AMP

FIG. 1. Effect of PMA on the cyclic AMP (cAMP) response to 10 μM isoproterenol (•); 100 μM norepinephrine (○), or 50 μM 2-chloroadenosine (□) in rat brain cortical slices. PMA was placed in the medium 15 min before addition of the stimulatory agent. Each point represents the mean of three experiments, each of which was performed in duplicate. In all cases, the SEM was < 15% of the mean.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 150–200 g (Timco, Houston, TX, U.S.A.) were housed five to a cage with free access to food and water. The animals were maintained on 12-h light-dark cycle.

Cyclic AMP analysis

Cyclic AMP accumulation was measured using a prelabeling technique (Shimizu et al., 1969). In brief, the animal was decapitated, and the brain or spinal cord was rapidly removed and placed into ice-cold Krebs-Ringer-bicarbonate buffer (pH 7.4) containing 118 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 11.1 mM glucose. Following dissection of the brain into regions, slices (350 µm) were prepared using a McIlwain tissue chopper and then preincubated for 15 min at 37°C in oxygenated (95% O₂, 5% CO₂) buffer. After preincubation, the slices were rinsed, placed into fresh buffer, and incubated for 1 h at 37°C with 0.1 μM [³H]adenosine. The labeled tissue was rinsed twice with buffer, and portions (~15 mg) placed into vials containing 440 μl of buffer and incubated for 10 min before addition of activators of adenylyl cyclase isoproterenol, 2-chloroadenosine, va-

soactive intestinal peptide (VIP), prostaglandin E₂ (PGE₂), norepinephrine, or forskolin. The phorbol esters were usually placed into the reaction mixture 15 min before addition of the activator. The samples were incubated for 10 min following exposure to the cyclic AMP stimulants, and the reaction was terminated by addition of 10% trichloroacetic acid (550 μl). The samples were homogenized and then centrifuged at 13,000 g for 10 min at 4°C, and total radioactivity was monitored in 50-μl samples of the supernatant. The remaining supernatant was assayed for [³H]cyclic AMP using the double column method of Salomon et al. (1974). The results are expressed as the percentage of total radioactivity present as cyclic AMP (percentage conversion).

Phosphorylation analysis

Rat brain cortical slices were preincubated for 60 min at 30°C in oxygenated Krebs-Ringer-bicarbonate buffer containing [³P]orthophosphate (1 mCi/ml) to equilibrate intracellular ATP pools. The tissue slices were rinsed twice

<table>
<thead>
<tr>
<th>Condition</th>
<th>Duration of PMA incubation before isoproterenol (min)</th>
<th>Cyclic AMP formation (% conversion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>—</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>—</td>
<td>0.56 ± 0.04</td>
</tr>
<tr>
<td>Isoproterenol + PMA</td>
<td>0</td>
<td>0.72 ± 0.04</td>
</tr>
<tr>
<td>Isoproterenol + PMA</td>
<td>5</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>Isoproterenol + PMA</td>
<td>10</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td>Isoproterenol + PMA</td>
<td>30</td>
<td>0.82 ± 0.04</td>
</tr>
<tr>
<td>Isoproterenol + PMA</td>
<td>60</td>
<td>0.80 ± 0.02</td>
</tr>
</tbody>
</table>

Rat brain cerebral cortical slices were exposed to PMA (100 μM) for the indicated interval before addition of isoproterenol (10 μM). In all cases, cyclic AMP accumulation was measured 15 min following exposure to the n-adrenergic agonist. Data are mean ± SEM values from four experiments, each of which was performed in duplicate. PMA alone was without effect on basal cyclic AMP accumulation.

*Significantly different from the 5-min preincubation condition (p < 0.05 by Student's two-tailed test).
TABLE 2. Influence of prolonged exposure to PMA on 2-chloroadenosine and 2-chloroadenosine plus PMA-stimulated cyclic AMP accumulation in rat brain cortical slices

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>PMA-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.05 ± 0.01</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>2-Chloroadenosine</td>
<td>1.62 ± 0.23</td>
<td>2.37 ± 0.24</td>
</tr>
<tr>
<td>2-Chloroadenosine + PMA</td>
<td>3.66 ± 0.30</td>
<td>2.37 ± 0.24</td>
</tr>
</tbody>
</table>

Rat brain cortical slices were incubated in the absence or presence of PMA (10 μM) for 3 h, rinsed twice with fresh buffer, and incubated an additional 10 min before addition of vehicle or 10 μM PMA for 15 min, at which time 2-chloroadenosine (50 μM) was added. Data are mean ± SEM values from three experiments, each of which was performed in duplicate.

with fresh buffer, portions (15–20 mg) were placed into tubes containing 300 μl of buffer, and PMA (10 μM) or vehicle was added to each after a 10-min preincubation. Following a 15-min exposure to PMA or vehicle, the slices were homogenized in a Potter-Elvehjem homogenizer in 50 volumes of buffer (pH 7.4) containing 100 mM NaF and 5 mM EGTA, and the homogenate was centrifuged at 48,000 g for 10 min. The resultant pellet was resuspended in the original volume of the NaF-EGTA buffer and centrifuged again at 48,000 g for 10 min. A portion of the membrane fraction (total protein, 60 μg) was added to an equal volume of buffer containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS), incubated for 5 min at 100°C, and subjected to electrophoresis in 10% (wt/vol) polyacrylamide gels according to the method of Laemmli (1970). The slab gel was stained with Coomassie Brilliant Blue and dried, and an autoradiogram was produced on Kodak XRP film by exposure for 5 h at −80°C in cassettes using DuPont Cronex Lightening Plus intensifier screens. Bio-Rad SDS-polyacrylamide gel electrophoresis (PAGE) low-molecular-weight standards were used as protein markers: lysozyme, M, 14,000; soybean trypsin inhibitor, M, 21,500; carbonic anhydrase, M, 31,000; ovalbumin, M, 45,000; bovine serum albumin, M, 66,200; phosphorylase b, M, 92,500.

In other experiments, membranes were prepared from brain slices incubated 45 min at 30°C in the absence or presence of PMA (10 μM) as described above. For analysis of endogenous protein kinase C activity, membrane fractions were incubated at 30°C in 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, 15 mM 2-mercaptoethanol, 200 μM phosphatidylethanolamine, 10 μM DAG, 2.0 mM MgCl₂, and 0.2 mM [γ-32P]ATP (108 cpm/μmol) in the absence or presence of 1 mM CaCl₂. Phosphorylation of control membranes by exogenously added protein kinase C (0.2 U/ml) plus PMA (1 μM) was also examined. In these experiments, cyclic AMP-dependent protein kinase activity was abolished by including the heat-stable protein inhibitor of this enzyme in the incubation medium (Whitehouse and Walsh, 1983). The reaction was terminated after 15 min by addition of SDS buffer, and phosphoprotein analysis was performed on 40-μg samples as described above. Protein content was determined by the method of Lowry et al. (1951).

Purification of protein kinase C

A partially purified preparation of protein kinase C was obtained from rat brain cerebral cortex using the method of Parker et al. (1984). One unit of activity was defined as the amount of enzyme required to phosphorylate 1 mmol of histone H1 in 1 min at 30°C.

Materials

[3H]Adenine (29 Ci/mmol) and [14C]cyclic AMP (44 mCi/mmol) were purchased from ICN Pharmaceuticals (Irvine, CA, U.S.A.). [32P]Orthophosphate (30 Ci/mmol) and [γ-32P]ATP (3,000 Ci/mmol) were purchased from Amersham Corp. (Chicago, IL, U.S.A.). Unlabeled cyclic AMP, (-)-isoproterenol, (−)-norepinephrine bitartrate, 2-chloroadenosine, PGE₂, quinacrine, phorbol esters, 1-oleoyl-2-acetyl-rac-glycerol (DAG), and phosphatidylserine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Forskolin was purchased from Calbiochem (San Diego, CA, U.S.A.) and VIP from Cambridge Research Biochemicals (Atlantic Beach, NY, U.S.A.). SDS-PAGE low-molecular-weight standards were obtained from Bio-Rad (Richmond, CA, U.S.A.). Ro 20-1724 was kindly donated by Dr. W. Burkhardt of F. Hoffmann-LaRoche (Basel, Switzerland).

FIG. 2. Concentration-response characteristics of 2-chloroadenosine (2-CL-ADO) in the absence (○) and presence (□) of 10 μM PMA in brain cortical slices. PMA was added 15 min before 2-CL-ADO. Each point represents the mean of three experiments, each of which was performed in duplicate. In all cases, the SEM was <15% of the mean. cAMP, cyclic AMP.
Accumulation was some twofold greater than that observed with isoproterenol. In the latter cases, the response to these agents was similar to that found with 3-amino-stimulated cyclic AMP formation (Fig. 3). With this agent, the phorbol ester approximately doubled the cyclic AMP accumulation in response to forskolin alone (Table 3). The more lengthy preincubation (60 min) had no effect on the potency of PMA to augment the second messenger response (data not shown).

The influence of prolonged exposure to PMA was also examined (Table 2). A 3-h preexposure to PMA increased only slightly the cyclic AMP response to 2-chloroadenosine alone. However, no additional enhancement of cyclic AMP accumulation was noted when 2-chloroadenosine was added in combination with PMA to phorbol-pretreated (3 h) slices.

A brief (15 min) preincubation with PMA had only a slight effect on the potency of 2-chloroadenosine to stimulate cyclic AMP production (Fig. 2). In the absence of PMA, the EC50 for 2-chloroadenosine was 18 μM, double that found in the presence of the phorbol ester. The concentration-response study also revealed that the maximal response to 2-chloroadenosine was some twofold greater when PMA was present as compared with 2-chloroadenosine alone (Fig. 2).

PMA enhanced (twofold) the second messenger response to a saturating concentration of PGE2 (Table 3). Likewise, PMA augmented the response to VIP, with the magnitude of the enhancement being dependent on the concentration of peptide. In the presence of 0.2 μM VIP, PMA (10 μM) increased the response over twofold, whereas the enhancement was only 40% with 1.0 μM VIP. PMA was also found to increase cyclic AMP accumulation in response to forskolin (Fig. 3). With this agent, the phorbol ester approximately doubled the response.

![Graph](image-url)
The augmenting response to PMA was regionally selective in the rat CNS (Table 4). PMA (10 μM) significantly increased 2-chloroadenosine-stimulated cyclic AMP accumulation in the cerebral cortex and hippocampus but had little effect in the pons-medulla, cerebellum, or spinal cord (Table 4). Higher concentrations (50 μM) of PMA yielded similar results. PMA had no significant effect on basal cyclic AMP levels in any of the regions examined (Table 4).

Neither EGTA (2.5 mM) nor quinacrine (200 μM) had any effect on the PMA-isoproterenol interaction in rat brain cerebral cortical tissue (Table 5). Likewise, the phosphodiesterase inhibitor Ro 20-1724 failed to modify the interaction, even though this substance increased cyclic AMP accumulation itself. Identical results were obtained with the phosphodiesterase inhibitor isobutylmethylxanthine (data not shown).

Of the four phorbol esters examined, only PMA and 4α-phorbol 12,13-dibutyrate significantly increased the cyclic AMP response to isoproterenol in cerebral cortical tissue (Table 6). With both phorbols, the response to isoproterenol was increased almost threefold. In contrast, neither 4α-phorbol nor 4α-phorbol 12,13-dideoxycacetate had any effect on isoproterenol-stimulated cyclic AMP production, even up to concentrations of 100 μM.

When the effect of PMA on protein phosphorylation in 32P-prelabeled slices was examined, it was found that the incorporation of 32P into trichloroacetic acid-precipitable proteins was increased 30–50% by the phorbol esters as compared with controls (data not shown). Of the large number of proteins present in the isolated membranes, relatively few were phosphorylated by endogenous cellular protein kinases (Fig. 4). Virtually all of the phosphoproteins showed some increase in 32P content following exposure of the brain slices to PMA. Analysis with a soft laser gel scanner indicated a widely varied (10–80%) increase in individual membrane protein phosphorylation.

The phosphorylation of membrane proteins by endogenous protein kinase C (Fig. 5, lanes A–D) was compared to that obtained in the presence of added C kinase (Fig. 5, lanes E and F). Maximal phosphorylation of the membrane fraction was observed after a 15-min incubation, as judged by analysis of total 32P incorporation into the trichloroacetate acid-precipitable proteins. The phosphoprotein patterns in membranes prepared from control and PMA-treated slices were qualitatively similar. The PMA-treated membranes incorporated ~30% less 32P under basal conditions (i.e., in the presence of 10 mM EGTA; Fig. 5.

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**TABLE 4. Regional distribution of the effect of PMA on 2-chloroadenosine-stimulated cyclic AMP accumulation in the rat CNS**

<table>
<thead>
<tr>
<th>Region</th>
<th>Basal</th>
<th>2-CL-ADO</th>
<th>PMA</th>
<th>2-CL-ADO + PMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral cortex</td>
<td>0.10 ± 0.01</td>
<td>1.47 ± 0.1</td>
<td>0.14 ± 0.02</td>
<td>3.13 ± 0.20^*</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.07 ± 0.01</td>
<td>1.57 ± 0.36</td>
<td>0.10 ± 0.02</td>
<td>2.72 ± 0.35^*</td>
</tr>
<tr>
<td>Pons-medulla</td>
<td>0.25 ± 0.09</td>
<td>1.52 ± 0.35</td>
<td>0.33 ± 0.09</td>
<td>1.89 ± 0.38</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>3.13 ± 0.19</td>
<td>3.48 ± 0.43</td>
<td>1.76 ± 0.19</td>
<td>4.02 ± 0.35</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>0.43 ± 0.09</td>
<td>1.04 ± 0.09</td>
<td>1.00 ± 0.16</td>
<td>1.40 ± 0.20</td>
</tr>
</tbody>
</table>

Cyclic AMP accumulation was examined in various regions of the rat CNS following addition of 2-chloroadenosine (2-CL-ADO) in the absence and presence of PMA (10 μM). PMA was added 15 min before 2-CL-ADO (50 μM). Data are mean ± SEM values from four experiments, each of which was performed in duplicate.

^* Significantly different from 2-CL-ADO alone (p ≤ 0.05 by Student's two-tailed t test).

**TABLE 5. Effects of EGTA, Ro 20-1724, and quinacrine on PMA enhancement of cyclic AMP accumulation in response to isoproterenol**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>-EGTA</th>
<th>-Ro 20-1724</th>
<th>-Quinacrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.06 ± 0.005</td>
<td>0.08 ± 0.01</td>
<td>0.19 ± 0.02</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>0.35 ± 0.02</td>
<td>0.33 ± 0.04</td>
<td>1.12 ± 0.13</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>Isoproterenol + PMA</td>
<td>0.93 ± 0.08</td>
<td>0.88 ± 0.12</td>
<td>2.00 ± 0.18</td>
<td>0.46 ± 0.10</td>
</tr>
</tbody>
</table>

EGTA (2.5 mM), Ro 20-1724 (25 μM), or quinacrine (200 μM) was added to rat brain cerebral cortical slices 10 min before PMA (10 μM) or buffer and 25 min before isoproterenol. Data are mean ± SEM values from three or four experiments, each of which was performed in duplicate.
lanes A and B). Incorporation of $^{32}$P into control membranes by endogenous protein kinase was increased 45-50% in the presence of calcium and phosphatidylserine (Fig. 5, lanes A and C). However, addition of 1 mM CaCl$_2$ did not significantly increase the degree of phosphorylation in PMA-treated membranes (Fig. 5, lanes B and D).

To establish that the calcium- and phospholipid-dependent phosphorylation observed in the brain membranes was catalyzed by protein kinase C, we examined the phosphorylation of control membranes by a partially purified preparation (Parker et al., 1984) of rat brain protein kinase C (Fig. 5, lanes E and F). The results indicated that some of the calcium-independent protein phosphorylation induced by endogenous protein kinase in control membranes (Fig. 5, lane A) was absent when an inhibitor of cyclic AMP-dependent protein kinase was added (Fig. 5, lane E), a result suggesting it may represent basal protein phosphorylation by a membrane-bound cyclic AMP-dependent kinase. Addition of protein kinase C (0.2 U/ml) and PMA (1 µM), which renders the enzyme calcium independent, enhanced phosphorylation some 2.5-fold (compare Fig. 5, lanes E and F), with the majority of phosphate incorporated into proteins that were also substrates for endogenous protein kinase C (arrows). No phosphorylation was noted when the membranes were exposed to PMA alone.

In a comparison of the phosphoprotein profiles resulting from PMA treatment of cerebral cortical slices and the exposure of brain membranes to purified C kinase, it was found that almost all proteins represented by the 20 bands incorporated some $^{32}$P under these conditions (Fig. 6). However, five of the six proteins incorporating the greatest amounts of radioactivity (15, 67, 77, 120, and 140 kilodaltons) during exposure of brain slices to PMA were phosphorylated to a similar extent when brain membranes were incubated with exogenous C kinase.

**TABLE 6.** Influence of various phorbol esters on isoproterenol-stimulated cyclic AMP accumulation in rat brain cerebral cortical slices

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyclic AMP formation (% conversion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>0.06 ± 0.005</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>Isoproterenol + PMA</td>
<td>1.14 ± 0.10</td>
</tr>
<tr>
<td>Isoproterenol + 4a-phorbol</td>
<td>1.20 ± 0.07</td>
</tr>
<tr>
<td>Isoproterenol + 4b-phorbol</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>Isoproterenol + 12:13-didecanoate</td>
<td>0.36 ± 0.03</td>
</tr>
</tbody>
</table>

Rat brain cortical slices were preincubated with one of the phorbol esters (10 µM) for 15 min before addition of isoproterenol (10 µM). Data are mean ± SEM values from four experiments, each of which was performed in duplicate.

**DISCUSSION**

This study confirms and extends previous reports that tumor-promoting phorbol esters augment drug- and neurotransmitter-induced second messenger production in a variety of tissues, including brain (Simantar and Sachs, 1982; Bell et al., 1985; Hollingsworth et al., 1985; Sugden et al., 1985). The discovery that PMA amplifies the second messenger response to a variety of substances, including noradrenergic receptor agonists. 2-chloroadenosine, VIP, and PGF$_2$ suggests that it modifies a component of the adenylate cyclase complex beyond the level of the receptor recognition site. This conclusion is supported by the finding that PMA enhances the cyclic AMP response to forskolin, an agent thought to stimulate directly the catalytic component of adenylate cyclase and perhaps N$_s$ (Seamon et al., 1981; Green and Clark, 1982). Thus, phorbol esters may enhance the function of N$_s$, or the catalytic unit or perhaps may facilitate the coupling between these proteins, amplifying the response to stimulation. Similar results have been reported for phorbol esters with respect to cyclic AMP production in S49 lymphoma cells, pinealocytes, and guinea pig brain (Bell et al., 1985; Hollingsworth et al., 1985; Sugden et al., 1985), although the present findings differ from those obtained with avian erythrocytes, in
which phorbol esters inhibit β-adrenergic-stimulated adenylate cyclase (Kelleher et al., 1984; Sibley et al., 1984).

The potency (EC_{50} = 1 μM) of PMA to amplify second messenger responses in brain is somewhat less than that observed for the modulation of activity in some systems (Ohmura and Friesen, 1985; Vicentini et al., 1985) but is similar to that found for other intact tissues (Labarca et al., 1984; Putney et al., 1984). The potency in tissue slices may, in part, be a function of the lipophilic nature of PMA, which enables this substance to accumulate in lipid compartments. Permeability barriers may also account for the time dependency of the response, with a greater augmenting response occurring when the tissue was exposed to the phorbol for several minutes before activation of adenylate cyclase. Alternatively, the delayed response to PMA may be indicative of a time-dependent modification of the second messenger system (i.e., phosphorylation).

Of particular interest was the finding that the augmenting response was eliminated following prolonged exposure to PMA. The small increase in cyclic AMP formation observed in response to 2-chloroadenosine following a 3-h preincubation with PMA may reflect a residual modification resulting from the initial exposure to the phorbol ester. However, when the tissue was incubated with PMA and 2-chloroadenosine following the prolonged preincubation with the phorbol ester, no further accumulation of cyclic AMP was observed. This contrasts with the results obtained with control tissue incubated for 3 h in the absence of the phorbol, for which combined exposures to PMA and 2-chloroadenosine caused a twofold enhancement in cyclic AMP formation relative to that observed with the adenosine analog alone. Thus, it is possible that protein kinase C, the presumed target of PMA, becomes "desensitized" during long-term exposure to the phorbol ester. Others have reported that both endogenous protein kinase C activity and phorbol ester binding capacity diminish in cell cultures following long-term incubation with phorbols (Collins and Rozengurt, 1984; Rodriguez-Pena and Rozengurt, 1984; Gainer and Murray, 1985; Wickremasinghe et al., 1985).

The magnitude of the response to PMA in brain tissue appears to be a function of the amount of cyclic AMP produced by the stimulating agent. At submaximizing concentrations of receptor agonist, PMA enhanced the response several-fold, whereas the degree
PHORBOL ESTERS AND BRAIN CYCLIC AMP

of augmentation was less as the concentration of agonist was increased. This was most apparent with the more efficacious activators of adenylate cyclase (2-chloroadenosine and VIP). This suggests a complex relationship between the degree of stimulation of the second messenger system and its capacity for enhancement by phorbol esters.

Protein kinase C is thought to be the primary site of action of phorbol esters (Niedel et al., 1983; Parker et al., 1984). That activation of protein kinase C was responsible for the augmenting response noted in the present study was indicated by the finding that only those phorbols known to interact with this enzyme enhanced cyclic AMP accumulation in the brain slice. Moreover, autoradiographic analysis revealed that exposure of [32P]phosphate-labeled slices to PMA increased the phosphorylation of membrane proteins. Furthermore, activation of endogenous C kinase with calcium, DAG, and phospholipid, although increasing protein phosphorylation in membranes prepared from control slices, had no effect on membranes obtained from tissue previously exposed to PMA. Presumably, this indicates that those sites normally available for 32P incorporation by endogenous phospholipid-dependent protein kinase had already been phosphorylated during the initial exposure to PMA. Finally, the involvement of protein kinase C in the response to the phorbols was also indicated by the finding that exposure of untreated membranes to a partially purified preparation of the enzyme resulted in a phosphoprotein profile similar to that observed following stimulation with calcium, DAG, and phospholipid. Thus, it would appear that phorbol esters stimulate C kinase in the rat brain slice under conditions in which cyclic AMP accumulation is augmented, a result suggesting that protein phosphorylation is an important mediator of this response.

The action of PMA on brain second messenger production is reminiscent of that reported for α-adrenergic and GABA\(_B\) receptor agonists (Daly et al., 1981; Karbon and Enna, 1985; Magistretti and Schorderet, 1985; Pilc and Enna, 1986). Thus, phorbols and α-adrenergic and GABA\(_B\) agonists all augment the response to a variety of cyclic AMP-coupled receptor agonists, an observation suggesting a postreceptor site of action. None is influenced by phosphodiesterase inhibitors, and all three have only a modest effect on the potency of the agonist to stimulate second messenger accumulation. Moreover, the regional distribution of the facilitating response in CNS tissue is quite similar for PMA and α-adrenergic and GABA\(_B\) agonists (Daly et al., 1981; Karbon and Enna, 1985), with the augmentation being greatest in the cerebral cortex and hippocampus. Because PMA binding sites have been identified throughout the mammalian CNS (Nagle et al., 1981; Murphy et al., 1983; Worley et al., 1985), it appears that the association between phorbol ester and the cyclic AMP-generating system varies among different brain areas. In addition, it is conceivable that the regional distribution of the augmenting response to PMA reflects regional differences in the permeability to the phorbol ester.

A major difference between the PMA-induced augmentation and that obtained with α-adrenergic and GABA\(_B\) agonists relates to their dependency on extracellular calcium. The response to PMA in the brain slice is not affected by EGTA, a substance known to eliminate the facilitating response to GABA\(_B\) and α-adrenergic agonists (Schwabe and Daly, 1977; Karbon and Enna, 1985). Moreover, whereas quinacrine, a nonselective inhibitor of phospholipase A\(_2\) (Snyder et al., 1984), reduces the augmenting response to α-adrenergic and GABA\(_B\) agonists (Duman et al., 1986), it has no effect on the response to PMA. These findings suggest that, in rat brain, the mechanism whereby α-adrenergic and GABA\(_B\) agonists augment cyclic AMP accumulation may differ somewhat from that of the phorbol esters.

The mechanism by which protein kinase C alters second messenger responses is unknown. Phorbol esters enhance secretory activity in some systems, making it possible that the cyclic AMP augmenting re-

![Diagram](image-url)
response is due to the action of a released substance rather than to a direct coupling between protein kinase C and the cyclic nucleotide system (Kaibuchi et al., 1982; Publicover, 1985). However, the fact that the PMA response is EGTA insensitive would seem to argue against the involvement of a calcium-dependent release mechanism. Alternatively, it is possible that C kinase catalyzes the phosphorylation of a protein involved in the regulation of second messenger production. It has recently been reported that protein kinase C phosphorylates the α-subunit of N, in platelet membranes, decreasing GTP-mediated inhibition of adenylyl cyclase (Jakobs et al., 1985; Katada et al., 1985). In the present study, phosphorylation of a 41,000-dalton protein, which may represent the α-subunit of N, was noted in brain tissue following the activation of C kinase. Regardless of the mechanism, these data indicate that protein kinase C may contribute in a significant manner to receptor-stimulated cyclic AMP production in brain and that, as in other tissues, this enzyme may be an important regulator of responses to receptor activation.

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REFERENCES


PJIORBOL ESTERS AND BRAIN CYCLIC AMP


PHORBOL ESTERS DOWN-REGULATE PROTEIN KINASE C IN RAT BRAIN CEREBRAL CORTICAL SLICES

Shirish Shenolikar, E. William Karbon*, and Salvatore J. Enna+

Departments of Pharmacology and of Neurobiology and Anatomy,
University of Texas Medical School at Houston, P.O. Box 20708, Houston, TX 77025

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The effect of phorbol esters on cyclic AMP production in rat cerebral cortical slices was studied using a prelabelling technique to measure cyclic nucleotide accumulation. Cholera toxin-stimulated cyclic AMP accumulation was enhanced approximately 2-fold by phorbol 12-myristate, 13-acetate (PMA) which alone had no effect on cyclic AMP production. The augmentation by PMA was maximal within the first hour of incubation, decreasing progressively thereafter. Protein kinase C activity was decreased 80-90% during a 3 hr exposure to PMA, as was 3H-phorbol 12,13-dibutyrate binding. Both phosphatidyl serine and arachidonic acid were found to enhance protein kinase C activity in a concentration-dependent manner, an effect that was attenuated by prolonged incubation of the brain tissue with PMA. The results indicate that exposure of brain slices to phorbol esters causes a down-regulation of rat brain protein kinase C, and that this modification corresponds with a decrease in the ability of PMA to augment cyclic AMP production, suggesting a functional relationship between the two systems in rat brain.

A number of components are associated with receptor-mediated changes in cyclic AMP production (1). Certain neurotransmitter receptors are directly coupled to adenylate cyclase by way of guanine nucleotide binding proteins (G), with some activating (through Gs) and others inhibiting (through Gi) adenylate cyclase activity (1). Other receptors are indirectly associated with second messenger production (2-4). In this case, receptor activation alone does not modify cyclic AMP production, although the response obtained during stimulation of other sites coupled to Gs is augmented (2-5). For example, while neither y-aminobutyric acid B (GABA-B) nor α-adrenergic receptor agonists alter basal levels of cyclic AMP in brain slices, both increase the amount of cyclic AMP accumulated during exposure of the tissue to β-adrenergic agonists, adenosine or vasoactive intestinal peptide (VIP). This augmenting action requires extracellular calcium ions (3-5), and is associated with the calcium-dependent enzyme, protein kinase C (6,7). This hypothesis was supported by the discovery that phorbol esters known to directly activate protein kinase C also augment transmitter-stimulated cyclic AMP production.
accumulation (8-11) and by the finding that the augmenting action of phorbol esters correlates
with protein kinase C-stimulated phosphorylation of brain proteins (10). Furthermore, prolonged
exposure to PMA resulted in a time-dependent attenuation of the augmenting effect of PMA on
cyclic AMP production.

The aim of the present study was to examine the effect of prolonged exposure to
phorbol esters on cellular protein kinase C activity and PMA-mediated augmentation of cyclic
AMP accumulation in rat brain slices. The results indicate that a 3 hr incubation of brain
tissue with phorbol esters decreases protein kinase C activity, phorbol ester binding, and
PMA-mediated augmentation of cyclic AMP accumulation. The findings point to the possibility
that protein kinase C is down-regulated under these conditions and suggest that this enzyme
contributes to the regulation of cyclic AMP production in brain.

MATERIALS AND METHODS

$^{3}$H-Adenine (29 Ci/m mole) and $^{14}$C-cyclic AMP (44 mCi/m mole) were purchased from
ICN, whereas $^{3}$H-phorbol 12,13-dibutyrate (10 Ci/m mole) and $^{32}$P-ATP (3000 Ci/m mole) were
obtained from Amersham Corporation. Phorbol 12-myristate, 13-acetate (PMA), phosphatidyl
sennse, diolein, and histone IIs were purchased from Sigma Chemical Co, DEAE-cellulose DE-52
from Whatman, histone H1 from Worthington Biochemicals, and cholera toxin from Calbiochem.

Cyclic AMP accumulation was measured using the prelabelling procedure of Shimizu
et al. (12). Rat brain cerebral cortical slices (350 x 350 mm) were incubated in an oxygenated
(95% O$_{2}$/ 5% CO$_{2}$) Krebs-Ringer bicarbonate buffer (4) containing 0.1 mM $^{3}$H-adenine for
1 hr at 37°C. The labelled tissue was rinsed twice and portions (15-20 mg wet weight) placed into vials
prior to incubation with PMA (10 $\mu$M) and/or cholera toxin (50 $\mu$g/ml). The reaction was
terminated by homogenizing the samples in 10% (w/v) trichloroacetic acid and the samples
centrifuged at 13,000 x g for 10 min. $^{3}$H-Cyclic AMP present in the supernatant was estimated
by the double column method of Salomon et al (13), using $^{14}$C-cyclic AMP to measure recovery.

In parallel experiments, unlabelled tissue slices that had been incubated in the presence or
absence of PMA were homogenized in 50 mM Tris-HCl buffer (pH 7.5), containing 250 mM
sucrose, 5 mM EGTA, 1 mM dithiothreitol, and 0.1% Triton X-100. The homogenate was
centrifuged at 100,000 x g for 45 min and the supernatant (2.5 mg protein) applied to a
deAE-cellulose column (1 x 3 cm) equilibrated in 10 mM Tris-HCl buffer (pH 7.5), containing 2
mM EDTA and 50 mM 2-mercaptoethanol. The column was washed extensively with the same
buffer prior to developing with a linear gradient of buffer containing 0 to 0.2 M NaCl (total
volume 25 ml). Protein kinase C activity was measured using histone IIs and histone H1 as
substrates. Protein kinase C activity and $^{3}$H-phorbol dibutyrate (PDB) binding were measured
using established procedures (14). The protein kinase C assays were routinely carried out in the
presence of excess heat-stable protein inhibitor of cyclic AMP dependent protein kinase.

RESULTS

A 3 hr exposure of rat brain cortical slices to cholera toxin (50 $\mu$g/ml) resulted in
a 14-fold increase in cyclic AMP accumulation (Table 1). Inclusion of PMA (10 $\mu$M) during the
final 15 min of incubation significantly increased the amount of cyclic AMP accumulated as

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TABLE 1. Cyclic AMP accumulation in rat brain cerebral cortical slices during incubation with cholera toxin and PMA

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>Cyclic AMP Accumulation (% Conversion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholera toxin alone (3 hr)</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>Cholera toxin + PMA (15 min)</td>
<td>1.05 ± 0.11*</td>
</tr>
<tr>
<td>Cholera toxin + PMA (30 min)</td>
<td>1.40 ± 0.10*</td>
</tr>
<tr>
<td>Cholera toxin + PMA (1 hr)</td>
<td>1.38 ± 0.08*</td>
</tr>
<tr>
<td>Cholera toxin + PMA (2 hr)</td>
<td>0.96 ± 0.09*</td>
</tr>
<tr>
<td>Cholera toxin + PMA (3 hr)</td>
<td>0.76 ± 0.05</td>
</tr>
</tbody>
</table>

In all cases, rat brain cerebral cortical slices were incubated with cholera toxin (50 μg/ml) for 3 hr. When present, PMA (10 μM) was added for the last 15 or 30 min, 1 hr, 2 hr, or during the entire 3 hr incubation period. Basal cyclic AMP accumulation was 0.05% throughout the 3 hr period. Each value represents the mean ± s.e.m. of 3 separate experiments, each of which was performed in duplicate. * p ≤ 0.05 compared to cholera toxin alone (two-tailed Student’s t-test).

Compared to cholera toxin alone, PMA-induced augmentation was concentration-dependent (EC50 = 1 μM), with 10 μM PMA yielding a maximal response (data not shown). Augmentation was observed only with those phorbol esters known to stimulate protein kinase C (data not shown), and was found to be maximal during the first hour of incubation, decreasing over the next 2 hr to the level obtained with cholera toxin alone (Table 1). The addition of PMA during a 1 or 2 hr exposure to cholera toxin also caused an augmentation of cyclic AMP accumulation, although the phorbol ester was much less effective when present for a 3 hr incubation period (Table 2).

TABLE 2. Influence of PMA on cholera toxin-induced accumulation of cyclic AMP in rat brain cerebral cortical slices

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>Cyclic AMP Accumulation (% Conversion)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without PMA</td>
</tr>
<tr>
<td>Cholera Toxin (1 hr)</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Cholera Toxin (2 hr)</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>Cholera Toxin (3 hr)</td>
<td>0.59 ± 0.03</td>
</tr>
</tbody>
</table>

Rat brain cerebral cortical slices were incubated with cholera toxin (50 μg/ml) alone or in the presence of PMA (10 μM) for 1, 2, or 3 hr, after which cyclic AMP accumulation was measured. Basal accumulation of cyclic AMP was 0.05% in all cases. Each value represents the mean ± s.e.m. of 3 separate experiments, each of which was performed in duplicate. * p ≤ 0.05 compared to corresponding value obtained in the absence of PMA (two-tailed Student’s t-test).
Fractionation of rat brain protein kinase C by DEAE-cellulose chromatography

The brain cytosol (100,000 x g supernatant) was applied to DEAE-cellulose as described in Methods. Portions (10 µl) of each fraction were assayed for protein kinase C activity by examining phosphorylation of histone III in the presence (solid line) and absence (dotted line) of calcium, diolein and phosphatidyl serine. Protein kinase activity in control tissue extract (circles) and extracts of tissue exposed to PMA for 3 hr (squares) are indicated.

Protein kinase C activity could be detected only after chromatography of the tissue cytosol (100,000 x g supernatant) on DEAE-cellulose (Figure 1). The ability of the enzyme to catalyze the phosphorylation of histone III was increased 15- to 30-fold in the presence of calcium (1 mM), diolein (20 µg/ml) and phosphatidyl serine (200 µg/ml). Control tissue displayed two peaks of histone kinase activity, one of which eluted at approximately 0.11 M NaCl. This fraction was sensitive to calcium and phosphatidyl serine, as expected for protein kinase C. However, only the first histone kinase peak was detected after a 3 hr exposure of the brain slices to PMA. As opposed to that found in 0.11 M NaCl, the activity of this enzyme (eluted at 0.05 M NaCl) was inhibited approximately 60% by calcium (1 mM) and phosphatidyl serine (200 µg/ml). Thus, whereas a 3 hr exposure to PMA reduced protein kinase C activity by 80-90%, the activity detected in the first peak of histone kinase was essentially unchanged.

Phosphatidyl serine stimulated protein kinase C activity in the eluted fractions in a concentration-dependent manner (Figure 2). The protein kinase activity from control tissue was stimulated maximally by concentrations of phospholipid greater than 200 µg/ml using histone H1 or histone III as substrates. The extent of activation in the presence of 1 mM CaCl2, diolein (20 µg/ml) and phosphatidyl serine (200 µg/ml) was greater using histone H1, being approximately 55-fold, which was similar to that observed with 1 µM PMA alone (data not shown). Under these conditions, two Ka values (5 µg/ml and 45 µg/ml) were found for...
phosphatidyl serine. In contrast to control tissue, fractions obtained from PMA-treated tissue phosphorylated histone H1 (with a 1.5 to 1.8-fold stimulation by Ca\(^{2+}\)/phospholipid) at maximal concentrations of phosphatidyl serine.

Like phosphatidyl serine, arachidonic acid also stimulated protein kinase C in control tissue, having an apparent \(K_a\) of 0.13 mM (Figure 3). Moreover, in comparison to the findings with phosphatidyl serine, enzyme from PMA-treated tissue failed to respond to arachidonic acid.

When \(^3\)H-phorbol dibutyrate (PDB) binding was examined in the presence of phosphatidyl serine (200 \(\mu\)g/ml), radioligand binding was found in precisely the same fractions as protein kinase C activity following ion-exchange chromatography (data not shown). The binding of \(^3\)H-PDB was stimulated by phosphatidyl serine in the absence of calcium ions, increasing approximately 5-fold in the peak fractions at saturating concentrations of the phospholipid. In contrast, \(^3\)H-PDB binding fractions obtained from PMA-treated tissue were unaffected by phosphatidyl serine, being identical to that observed in control slices in the absence of the phospholipid. Moreover, PMA (10 \(\mu\)M) displaced very little (< 20%) of the total isotope bound to fractions from tissue exposed for 3 hr to the phorbol ester (Figure 4).
Figure 3. Activation of rat brain protein kinase C by arachidonic acid

Histone H1 phosphorylation was activated by various concentrations of arachidonic acid in the presence of 1 mM CaCl2 and diolein (20 µg/ml). Each point represents the mean ± s.e.m. of 3 experiments. Closed circles represent the activation of the control fraction and squares represent the fraction from PMA-treated tissue.

DISCUSSION

Recent reports have indicated that PMA enhances the ability of a variety of receptor agonists to increase intracellular levels of cyclic AMP, suggesting that PMA modifies a post-receptor constituent of the adenylate cyclase system. The fact that FMA augments cyclic

Figure 4. Effect of PMA exposure on [3H]Phorbol 12,13-dibutyrate binding to rat brain protein kinase C

Rat brain cerebral cortical slices were incubated with PMA for 3 hr after which the peak fractions of protein kinase C activity obtained from DEAE-cellulose were analysed for phosphatidylinositol-stimulated [3H]PDB binding. Portions (50 µg) of the peak fraction, representing approximately 3.5 µg protein, were incubated for 15 min at 30°C with 3H-PDB (10 nM). Specific binding was defined as the difference between total binding and that observed in the presence of a saturating 100 nM concentration of unlabelled PMA. Each point represents the mean ± s.e.m. of 3 experiments, each of which was performed in duplicate. Control fractions (closed circles) and fractions from the PMA-treated tissue (squares) were assayed.
AMP production in response to forskolin (10, 14), a diterpine that directly stimulates the catalytic subunit of adenylate cyclase and perhaps Gs (15,16), would seem to support this conclusion. Moreover, as demonstrated in the present study, PMA also augments the cyclic AMP response to cholera toxin, an agent that promotes second messenger accumulation by ADP-ribosylating Gs, lending further support to the notion that PMA influences some component of the adenylate cyclase system beyond the receptor recognition site. The potency of PMA (EC50 = 1 μM) in this regard was similar to that observed previously with intact tissue (10, 17). The capacity of the partially purified rat brain protein kinase C to be fully activated in vitro by 1 μM PMA suggests that the higher concentration required with intact tissue may be due to a limited penetration of the phorbol into the slice preparation (10).

The major finding of the present study was that prolonged exposure of rat brain slices to PMA reduces the ability of the phorbol ester to augment cholera toxin-stimulated cyclic AMP accumulation. The decline in the augmenting response to PMA does not appear to be due to a decrease in the capacity of adenylate cyclase to synthesize cyclic AMP since cholera toxin-stimulated second messenger accumulation was unaffected by prolonged exposure to PMA. Moreover, previous work has demonstrated that the cyclic AMP response to 2-chloroadenosine is not modified by a long-term incubation of the rat brain tissue slice with phorbol esters (10). Prolonged exposure of cells to phorbol esters has been reported to diminish cellular protein kinase C or total phorbol binding (18-22), and a down-regulation of brain protein kinase C following a 3 hr incubation with PMA has been previously suggested (10).

In the present study, a maximal extraction of protein kinase C was achieved by homogenizing brain tissue with 5 mM EGTA and 0.1% Triton X-100. Extensive washing of the particulate fraction with 1% Triton X-100 failed to yield additional protein kinase C, indicating a complete liberation of the enzyme by this treatment. Analysis of the extract fractionated on DEAE-cellulose suggested a selective time-dependent decrease in calcium- and phosphatidyl serine-dependent protein kinase activity following prolonged exposure to PMA. Tissue treated with PMA retained less than 20% of its protein kinase C activity when assayed at all concentrations of phosphatidyl serine or arachidonic acid, indicating that the loss of activity was not due to an alteration in the Kₐ of the allosteric regulators of the enzyme. The finding that phosphatidyl serine-stimulated ³H-PDB binding was reduced to a similar extent as protein kinase C activity confirms that the primary cellular receptor for the phorbol esters is no
longer available (13, 21). Immunological analysis of the absolute amount of protein kinase C, as undertaken by Ballester and Rosen (25), will be required to establish whether this change is due to a loss of enzyme (20-24) or to a modification in enzyme activity.

The present findings represent the first demonstration of a phorbol ester-stimulated down-regulation of protein kinase C in a tissue preparation. The results indicate a key role for this enzyme in the augmentation of neurotransmitter-stimulated cyclic AMP accumulation in brain, implying an association between protein kinase C and the adenylate cyclase system in the regulation of receptor-mediated responses.

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We thank Mr. Jeffery Langston for his excellent technical assistance. This work was supported in part by a Biomedical Research Support Grant from the University of Texas Medical School (S.S.), by a U.S.P.H.S. Research Scientist Development Award (MH-00501) to S.J.E. and by a U.S. Air Force contract.

REFERENCES

THIP, A GABA AGONIST, ATTENUATES ANTINOCICEPTION IN THE MOUSE
BY MODIFYING CENTRAL CHOLINERGIC TRANSMISSION

Stevin H. Zorn and S. J. Enna

Departments of Pharmacology and of Neurobiology and Anatomy
The University of Texas Medical School at Houston
P. O. Box 20708, Houston, Texas 77025

Running Title: THIP blocks GABA analgesia

* Present address: Nova Pharmaceutical Corporation, 5210 Eastern Avenue, Baltimore, Maryland 21224. To whom reprint requests should be addressed.
ABSTRACT

Summary - The effect of THIP, a direct-acting GABA receptor agonist, on the antinociceptive response to a variety of agents was examined using the mouse tail-immersion assay. THIP alone produced an antinociceptive response at lower doses (5 mg/kg) but was ineffective at doses exceeding 10 mg/kg. Treatment with THIP (15 mg/kg) was found to block the antinociceptive response to a GABA uptake inhibitor, an inhibitor of GABA transaminase, a direct-acting GABA receptor agonist and to a cholinesterase inhibitor. In contrast, THIP had no effect on the antinociceptive responses to morphine, clonidine or oxotremorine. The results indicate that higher doses of THIP reduce cholinergic activity in a pathway important for mediating the antinociceptive action of GABAergic drugs and physostigmine.
Compounds that facilitate \( \gamma \)-aminobutyric acid (GABA) neurotransmission are known to induce antinociception in laboratory animals and analgesia in man (Kjaer and Nielson, 1983; Lindeburg, Folsgard, Silleson, Jacobsen, Kehlet, 1983; Sawynok, 1984; Vaught, Pelley, Costa, Setler, and Enna, 1985; Zorn and Enna, 1985a). This action is shared by THIP (4,5,6,7-tetrahydroisoxazolo (5,4-c) pyridin-3-ol) and baclofen (\( \beta \)-p-chorophenyl-GABA), direct-acting GABA receptor agonists (Christensen and Larsen, 1982; Hill, Maurer, Buescher, Roemer, 1981; Levy and Proudfit, 1977; Vaught, et al., 1985), as well as by substances that indirectly augment GABAergic transmission by inhibiting the catabolism (\( \gamma \)-acetylenic GABA) or re-uptake (SKF 100330A) of this amino acid (Bucket, 1980; Sawynok and Dickson, 1983; Zorn and Enna, 1985a). Studies have suggested that GABAergic-induced antinociception is mediated by an action at supraspinal sites and is secondary to the activation or disinhibition of central cholinergic pathways (Kendall, Browner, and Enna, 1982; Levy and Proudfit, 1979; Liebman and Pastor, 1980; Proudfit and Levy, 1978; Retz and Holaday, 1984; Reyes-Vazquez, Enna, and Dafny, 1986; Zorn and Enna, 1985a&b). While GABAergic drugs are active in tests predictive of opiate-like analgesia, their antinociceptive action is not blocked by naloxone (Hill et al., 1981; Kendall, et al., 1982; Levy and Proudfit, 1979; Sawynok and LaBella, 1984; Vaught et al., 1985; Zorn and Enna, 1985a), whereas centrally-active muscarinic receptor antagonists are effective in this regard (Kendall et al., 1982; Vaught et al., 1985; Zorn and Enna, 1985a). Although these GABAergic agents are sedating, data indicate that the
antinociceptive action is unrelated to central nervous system depression (Kendall et al., 1982; Levy and Proudfit, 1977; Zorn and Enna, 1985a).

Unlike other GABAergic drugs, the antinociceptive response to THIP displays a bell-shaped dose-response curve (Kendall et al., 1982; Zorn and Enna, 1985b), producing a significant antinociceptive response at lower doses, but having no effect at doses exceeding 10 mg/kg (Kendall et al., 1982; Zorn and Enna, 1985b). This suggests that higher doses of THIP have an effect that counteracts its own antinociceptive action. The present study was undertaken to examine this property by studying the interaction of THIP with a variety of antinociceptive agents. The results indicate that THIP is capable of attenuating the antinociceptive response to other GABAergic drugs and to physostigmine, suggesting that at higher doses it may reduce central cholinergic activity in pathways important for mediating the action of these substances.

METHODS

Animals

Male albino CF-1 mice (30-35g, Charles River, Wilmington, MA) were housed on a 12 hr light/dark cycle with access to food and water ad libitum. Except for baclofen, which was dissolved in 0.05 M HCl, the drugs were dissolved and administered (5 μl/g, i.p.) in distilled water. Control animals received an equivalent volume of vehicle. In some experiments the animals were injected with THIP (5-20 mg/kg) in combination with SKF 100330A (30 mg/kg), baclofen (20 mg/kg), γ-acetylenic GABA (GAG) (150 mg/kg), morphine (15 mg/kg), clonidine (1.5 mg/kg), physostigmine
(0.4 mg/kg), oxotremorine (0.05 - 0.1 mg/kg) or bicuculline (1.0 mg/kg). In these cases the drugs were administered to allow for measurement of the antinociceptive response at a time when both agents are known to produce maximal effects (Vaught et al, 1985; Zorn and Enna, 1985 a&b; Zorn and Enna, 1985b). GAG, baclofen or bicuculline were injected 90, 30 or 5 min prior to THIP, respectively, and nociception measured 30 min later. Clonidine, SKF 100330A, and physostigmine were administered 15 min after THIP, with nociception tested 15 min later. In one group of experiments, bicuculline was injected 10 min after SKF 100330A (25 min after THIP) and nociception tested 5 min later. Morphine and oxotremorine were administered concurrently with THIP, 30 min prior to analysis.

Antinociceptive activity was measured using the tail-immersion assay (Zorn and Enna, 1985a&b). Each animal was restrained in a specially designed plastic holder to allow free movement of the tail, the distal portion (1-2 cm) of which was immersed into a 50 ± 0.25°C water bath. Nociception was quantified by measuring the time elapsing between immersion and an attempt by the animal to remove the tail from the water bath. A maximum antinociceptive response was arbitrarily defined as a failure to withdraw the tail within 30 sec. Each animal was tested prior to drug administration to establish the control response time. Animals receiving only vehicle responded no differently from those subsequently used for drug treatment. All data are expressed as a % of the control response. Data were evaluated by an ANOVA and a lowest significant difference (LSD).
analysis, or by a Student's t-test. Differences were considered statistically significant when $P < 0.05$.

The following drugs were generously donated: THIP, V. Christensen, H. Lundbeck and Co., Copenhagen, Denmark; baclofen, CIBA-GEIGY, Summit, N.J.; SKF 100330A, Dr. W. E. Bondinell, Smith, Kline and French Laboratories, Philadelphia, PA; and γ-acetylenic GABA, Merrill International, Strasbourg, France. Oxotremorine, clonidine, physostigmine and bicuculline were purchased from Sigma Chemical Co., St. Louis, MO., and morphine sulphate from Penick Corporation, Garden City, NJ.

RESULTS

At 5 mg/kg, THIP alone increased the response latency in the mouse tail-immersion assay approximately 90%, whereas at higher doses (10 and 15 mg/kg) an antinociceptive response was no longer detectable (Fig. 1). Identical data were obtained with animals pretreated with bicuculline (1 mg/kg) 5 min prior to THIP (data not shown). The GABA uptake inhibitor SKF 100330A (30 mg/kg) was more efficacious than THIP, tripling the latency to response in the tail-immersion assay (Fig. 1). When mice received THIP (5 mg/kg) 15 min prior to the SKF compound, there was a significant reduction in the antinociceptive response to the uptake inhibitor, with the latency being similar to that found with this dose of THIP alone. Moreover, prior administration of a higher dose (15 mg/kg) of THIP completely abolished the antinociceptive response to the SKF compound (Fig. 1), an effect that was not influenced by injecting the animals with bicuculline either 5 min prior to THIP or 5 min prior to measurement of the
The higher dose of THIP also blocked the antinociceptive response to baclofen, and substantially reduced that associated with GAG (Fig 2). In addition, THIP pretreatment reduced the antinociceptive response to physostigmine, a cholinesterase inhibitor (Fig 3). Thus, when THIP (15 mg/kg) was administered 15 min prior to a dose (0.4 mg/kg) of physostigmine that by itself increased the response latency some 5-fold, it completely prevented the antinociceptive effect produced by this compound. In contrast, THIP did not influence the antinociceptive action of oxotremorine, a direct-acting muscarinic receptor agonist (Fig 3), nor did it modify the responses to morphine or clonidine (data not shown). Negative data were obtained with both maximal and submaximal doses of oxotremorine.

A dose-response study revealed that THIP blocked the antinociceptive response to physostigmine over a very narrow range (Fig 4). Whereas no significant inhibition was noted at a 10 mg/kg dose of THIP, the blockade was maximal at 12.5 mg/kg and above (Fig. 4).

DISCUSSION

The major finding of this study is that THIP blocks antinociceptive responses produced by GABAergic drugs and physostigmine. This discovery was somewhat surprising since THIP itself is known to be an antinociceptive agent (Christensen and Larsen, 1982; Hill et al, 1981). However, as reported previously, although THIP induced a significant antinociceptive response at
lower doses, it is inactive when administered at doses greater than 10mg/kg (Kendall et al, 1982; Zorn and Enna, 1985b). These data suggest that the higher doses of THIP reduce the nociceptive threshold in the mouse tail-immersion assay, or that THIP is capable of reversing its own action. The effect of THIP on morphine- and clonidine-induced antinociception was studied to test the former possibility. Inasmuch as THIP was unable to reduce the antinociceptive responses to these agents, it would appear that a generalized effect on nociceptive threshold cannot explain its action at higher doses. Moreover, although THIP, like other GABAergic drugs, depresses central nervous system function (Christensen, Svendsen, and Krogsgaard-Larsen, 1982), it has been shown previously that this cannot account for the antinociceptive response to these agents (Kendall et al, 1982). This is confirmed in the present study by the finding that doses of THIP (10-20 mg/kg) causing overt signs of sedation failed to modify the nociceptive threshold in the tail-immersion assay.

Given the negative findings with respect to a generalized effect on nociception, experiments were undertaken to examine whether THIP selectively modifies the antinociceptive response to other GABAergic drugs. At the highest dose tested (15 mg/kg), THIP completely blocked the antinociceptive action of baclofen, a selective GABA<sub>B</sub> receptor agonist (Hill and Bowery, 1981; Karbon, Duman, and Enna, 1984), GAG, a GABA transaminase inhibitor (Buckett, 1980), and SKF 100330A, a GABA uptake inhibitor (Ali, Bondinell, Dandridge, Frazee, Garvey, Girard, Kaiser, Ku, Lafferty, Moonsammy, Oh, Rush, Setler, Stringer, Venslavsky, Volpe, Yunger, and Zirkle, 1985; Yunger, Fowler,
Zarevics, and Setler, 1984; Zorn and Enna, 1985a). Pretreatment with an antinociceptive dose (5 mg/kg) of THIP reduced the response to the uptake inhibitor to that found with THIP alone. This suggests that THIP may be acting as a partial agonist at those GABA receptors mediating the antinociceptive response. Indeed, biochemical studies have indicated that THIP may be a partial agonist for the GABA/benzodiazepine receptor complex (Braestrup and Squires, 1977; Falch and Krogsgaard-Larsen, 1982; Hosli, Krogsgaard-Larsen and Hosli, 1985; Karobath and Lippitsch, 1979). However, a partial agonist action cannot totally explain the present findings since, at higher doses, THIP abolished its own antinociceptive response, as well as that induced by other GABAergic drugs.

It is conceivable that the inhibitory action of THIP on GABAergic-induced antinociception may in part be secondary to an influence on some pathway or system distal to the GABAergic neurons that regulate nociception. Since the central cholinergic system is known to play a crucial role in mediating the antinociceptive response to GABAergic drugs (Kendall et al, 1982; Vaught et al, 1985; Zorn and Enna, 1985a), the effect of THIP on the antinociceptive responses to two types of cholinomimetics was tested. The finding that THIP abolished the antinociceptive response to physostigmine, a cholinesterase inhibitor, suggests that it reduces cholinergic activity in a system capable of mediating antinociceptive responses. However, the fact that THIP failed to block the antinociceptive action of oxotremorine, a direct-acting muscarinic receptor agonist,
indicates that THIP does not act at the level of the muscarinic receptor. This accords with earlier studies indicating that THIP has no appreciable affinity for muscarinic binding sites in brain (Kendall et al, 1982). Since physostigmine prolongs the action of acetylcholine by inhibiting its hydrolysis, the findings suggest that, at higher doses, THIP may reduce the release of this neurotransmitter substance. Indeed, in vitro studies have suggested that THIP inhibits the electrically-induced release of acetylcholine from rat brain slices (Supavilai and Karobath, 1985). Such an effect could explain why the antinociceptive efficacy of THIP is less than for other GABAergic agents since it has opposing actions on cholinergic transmission. Whereas at lower doses THIP is capable of enhancing cholinergic activity to yield an antinociceptive response (Kendall et al, 1982), at higher doses the inhibitory action on cholinergic transmission may predominate, attenuating its own antinociceptive action as well as the response of agents requiring the involvement of this cholinergic pathway.

Although it has been proposed that THIP is a direct-acting agonist at GABA receptors (Christensen et al, 1982), recent studies suggest that it may be selective for a subpopulation of these sites (Falch and Krogsgaard-Larsen, 1982; Hosli et al, 1985). Indeed, the inability of bicuculline to modify either the antinociceptive response to THIP or its ability to antagonize the action of SKF 100330A would seem to confirm that these actions of THIP are unrelated to bicuculline-sensitive GABA receptors. However, given the necessity of using subconvulsant doses of bicuculline to test this hypothesis, it remains possible
that the amount of antagonist administered was insufficient for blocking the action of THIP (Vaught et al., 1985). Thus it is impossible to conclude whether either of these actions of THIP is mediated by bicuculline-sensitive sites. Nevertheless, the present findings suggest that at least some THIP-sensitive GABA receptors may be located on central cholinergic neurons. These results also support the notion of functionally distinct GABA receptor systems, and provide further information with regard to the antinociceptive properties of GABAergic drugs.

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FIGURE LEGENDS

Fig. 1 Effect of THIP on the antinociceptive action of SKF 100330A, a GABA uptake inhibitor. Antinociceptive responses were quantified by the tail-immersion assay 30 min after the administration of THIP (5 or 15 mg/kg) or 15 min after SKF 100330A (30 mg/kg). Animals receiving both THIP and SKF 100330A were injected with THIP 15 min prior to the SKF compound, with nociception assessed 15 min later. The height of each bar represents the mean % of control reaction time ± S.E.M. of 7-30 animals. The doses (mg/kg) for THIP are indicated in brackets.
* $P < 0.05$ compared to THIP alone (ANOVA, LSD analysis).

Fig. 2 Effect of THIP (15 mg/kg) on the antinociceptive responses to baclofen and $\gamma$-acetylenic-GABA (GAG). Antinociception was assessed using a tail-immersion procedure 60 or 120 min after the administration of baclofen or GAG, respectively. For combined studies, THIP was injected either 30 (baclofen) or 90 (GAG) min after these drugs and nociception quantified 30 min later. The height of the bars represents the mean % of control reaction time ± S.E.M. of 7-8 animals.
* $P < 0.05$ (Student's t-test).

Fig. 3 Effect of THIP on the antinociceptive response to oxotremorine (OXO) and physostigmine (PHY). Nociception was examined 30 min after OXO and 15 min
after PHY by the tail-immersion assay. In combination experiments the animals were injected with THIP (15 mg/kg) 15 min prior to PHY or concurrently with OXO. Each bar represents the mean % of control reaction time ± S.E.M. of 7-14 animals.

* P < 0.05 compared to corresponding control (Student's t-test).

**Fig. 4** Dose-response characteristics of THIP on the antinociceptive action of physostigmine (0.4 mg/kg) in the mouse tail-immersion assay. THIP was administered 15 min prior to physostigmine and nociception quantified 15 min later. Each point represents the mean % of control reaction time ± S.E.M. of 6 animals.

* P < 0.05 compared to control (Student's t-test).
γ-AMINOBUTYRIC ACID (GABA) RECEPTORS AND THEIR ASSOCIATION WITH BENZODIAZEPINE RECOGNITION SITES

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S. J. Enna and Hanns Mohler

* Departments of Pharmacology and of Neurobiology and Anatomy
University of Texas Medical School at Houston
P.O. Box 20708, Houston, Texas 77025
(713-792-5734)

** Pharmaceutical Research Department
F. Hoffmann-LaRoche, Ltd., CH-4002 Basel, Switzerland
(61-271122, Ext. 3762)

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To whom correspondence and reprint requests should be addressed.
INTRODUCTION

A number of amino acids are considered neurotransmitter candidates (34). Those receiving the most intense scrutiny have been glutamic and aspartic acids, compounds that induce excitatory responses in the mammalian central nervous system, as well as γ-aminobutyric acid (GABA) and glycine, which are classified as inhibitory neurotransmitters. The majority of information relating to amino acid neurotransmitters has derived from studies with GABA since more is known about its synthesis, metabolism, and pharmacological characteristics. Thus, investigators have at their disposal agents that inhibit GABA degradation and re-uptake, as well as direct-acting GABA receptor agonists and antagonists (35,43). These tools have made it possible to characterize more fully the properties of GABAergic synapses as compared to other amino acid substances.

Interest in GABA has also been stimulated by suggestions that this transmitter system may be affected in a variety of central nervous system disorders such as Huntington's Disease, epilepsy and Parkinson's Disease (59,81,89). Moreover, manipulation of GABAergic transmission may have a beneficial effect in the treatment of anxiety and depression and it has been hypothesized that GABAergic drugs may be useful in the management of schizophrenia (90).

Given these findings, the GABAergic system is considered a prime target for new psychotherapeutic agents. However, the extensive distribution of GABA in the mammalian brain and spinal cord (45) has hindered the development of GABAergic drugs since they tend
to have a generalized effect on central nervous system function. Accordingly, to develop more selective agents it is necessary to identify differences among the various GABAergic synapses in brain, such as pharmacologically and functionally distinct GABA receptors. This approach has met with some success in that two distinct GABA receptors have now been proposed (10,36). These sites, designated GABA_A and GABA_B, differ with regard to their substrate specificity, ionic characteristics, and biochemical properties. One of the more important distinctions is that the GABA_A receptor is associated with the neuronal membrane recognition site for benzodiazepines, a discovery that has provided new insights into the mechanism of action of this drug class (108). Although less is known about GABA_B receptors, data suggest they serve to modulate receptor function for other neurotransmitters (65). The aim of the present report is to summarize current concepts relating to the pharmacological and functional properties of GABA receptor sites and their relationship to benzodiazepines. Particular emphasis is placed on evaluating these data from the perspective of psychopharmacology. Readers desiring a more detailed discussion of individual topics are urged to consult any of a number of monographs and reviews (26,37,39,41,47,108).

GABA RECEPTORS

The initial data suggesting a neurotransmitter role for GABA in mammalian systems was derived from electrophysiological studies (30). These findings indicated that GABA causes a hyperpo-
larizing response in virtually all neuronal cells when applied at sufficient concentrations. Because of this apparent lack of selectivity, early investigators were reluctant to assign a neurotransmitter role for GABA. This attitude changed with the discovery of agents (bicuculline and picrotoxin) capable of inhibiting selectively the hyperpolarizing response to GABA (28). The finding that both of these compounds are rather potent convulsants confirmed the suggestion that GABA serves as an inhibitory transmitter substance.

Electrophysiological studies also revealed that the hyperpolarizing response to GABA is due to an increase in chloride conductance (29,103). Since for most neurons the extracellular concentration of chloride exceeds that in the cytoplasm, GABA receptor activation facilitates the entry of this anion, increasing the firing threshold of the cell.

During the past decade ligand binding assays have made it possible to obtain a more detailed knowledge of the biochemical and pharmacological properties of GABA receptors (40). These investigations have indicated that GABA receptor binding sites are located in virtually all regions of the central nervous system, from the retina to the spinal cord. Ligand binding data have also revealed that the brain contains a number of kinetically distinct GABA receptors (44,46). While it was initially believed that the site possessing the highest affinity for GABA normally mediates the response to this transmitter, more recent studies have suggested that a lower affinity receptor may be most closely associated with the effector system (3,17,66,104). These initial electrophysiological and biochemical studies demonstrated
that the GABA receptor consists of at least two basic components: the GABA receptor recognition site and an associated ion channel (Figure 1).

A number of substances have been found to selectively influence the GABA receptor components (Figure 1). Direct-acting agonists for the GABA recognition site include muscimol, THIP (4,5,6,7-tetrahydroisoaxazolo[5,4-c]pyridine-3-ol), and isoguvacine (69). Competitive antagonists for this site are bicuculline, securinine, and SR-95103 (2-[carboxy-3'-propyl]-3-amino-4-methyl-6-phenylpyridazinium) (6,20,44). In general, GABA receptor agonists are central nervous system depressants, muscle relaxants, and possess some antinociceptive properties, whereas the receptor antagonists are convulsants (38).

Drugs have also been found that directly modify the functioning of the chloride ion channel (Figure 1). Included in this group are picrotoxin, TBPS (tert-butylbicyclopentosphorothionate), and TBOB (4-tert-butyl-1-[4-cyanophenyl]-bicycloorthocarboxylate) (19,99,105). Selective binding sites for these chloride channel agents have been described using radiolabeled derivatives. All three appear to bind to the same site and, under the proper conditions, all can influence the binding of GABA receptor agonists, indicating an allosteric relationship between the chloride channel and recognition site components (87,97). Inasmuch as these substances are convulsants, they are considered chloride channel blockers.

A variety of centrally-active drugs influence ligand attachment to the chloride channel binding site, including seda-
tive-hypnotics, anticonvulsants and some non-benzodiazepine anxiolytics (61,106). While none of these agents competitively interact with the picrotoxin site, their binding component seems to be more intimately associated with the chloride channel than with the GABA receptor recognition site. Such findings indicate that GABA receptor function can be pharmacologically manipulated in a variety of ways.

THE BENZODIAZEPINE RECOGNITION SITE

A major advance in understanding the mechanism of action of anxiolytics was the discovery of benzodiazepine binding sites in the mammalian central nervous system (77,98). Evidence that these sites mediate the responses to this drug class was provided by the finding that the relative affinities of benzodiazepines for this site paralleled their relative potencies in behavioral tests predictive of anxiolytic and anticonvulsant activity (12,78). A direct association between the benzodiazepine binding component and GABA receptors was suggested from biochemical experiments showing that activation of the GABA recognition site enhances the affinity of benzodiazepines receptors in brain (25,66,102). Autoradiographic studies have confirmed this affiliation by demonstrating that benzodiazepine binding sites are generally found in close proximity to GABAergic synapses (79,80). These data confirm earlier electrophysiological results indicating that the benzodiazepines enhance GABAergic transmission (21-23,51,54,56,73,100). Of particular importance was the discovery that most, if not all, benzodiazepine binding sites are associated with GABA receptors (79,94). However, additional GABA A
receptor sites appear to exist which are devoid of a benzodiazepine component (107); it is unknown whether these sites are located synaptically. The effects of benzodiazepines contrast with the barbiturates which enhance GABAergic transmission by acting at chloride channels as well as depressing excitatory transmission thereby exerting a more generalized effect on nervous system function (54,83,106).

Electrophysiological studies have revealed that the benzodiazepines increase the probability of opening of chloride channels in response to GABA (100), an action which may account for the pharmacological and therapeutic actions of these drugs (2,55). The dose-response curve for the GABA-induced change in chloride conductance is shifted to the left in the presence of benzodiazepines, with no change in the maximal response (23). This indicates that benzodiazepines enhance GABA receptor function only at synapses where the GABA concentration is insufficient to open all available chloride channels, but do not promote receptor function beyond that which can be obtained with GABA itself. This may explain why the benzodiazepines have a more favorable therapeutic index as compared to other central nervous system depressants which, at high doses, may depress neuronal function beyond the normal range.

When considering a general mechanism of action of benzodiazepines it is important to recall that unless the receptor is activated by GABA the benzodiazepines are ineffective (23,54,56). This suggests that benzodiazepines enhance GABA receptor function only at GABAergic synapses while having no
influence on extrasynaptic GABA sites. This may explain the more selective actions of the benzodiazepines as compared to drugs acting directly at the GABA recognition site since the latter may stimulate or inhibit GABA sites regardless of their neuroanatomical location.

Three types of benzodiazepine receptor ligands have been identified (Figure 1). Agents such as diazepam, chlorodiazepoxide, and flunitrazepam are classified as benzodiazepine receptor agonists since they enhance GABA receptor function. Also included in this group are non-benzodiazepine tranquilizers (53) such as certain triazolopyridazines, e.g. CL 218872 (71), cyclopyrrolones, e.g. zopiclone (7), phenylquinolinones, e.g. PK 8165 (70), pyrazoloquinolinones, e.g. CGS 9896 (112), and some β-carbolines, e.g. ZK 93423 (72). Other substances, such as β-carboline carboxylate ethyl ester, produce effects opposite to those found with the benzodiazepine receptor agonists (14,15,88), and are therefore referred to as inverse agonists. Binding studies indicate that agonists and inverse agonists attach to the same or overlapping sites on the benzodiazepine binding component (75). This was also demonstrated by the discovery of a third class of ligand, benzodiazepine receptor antagonists (5,50,52,60,75). These are represented by RO 15-1788, an imidazobenzodiazepinone (60). RO 15-1788 is largely devoid of pharmacological effects but competitively interacts at the benzodiazepine binding site to block the actions of either the receptor agonists or inverse agonists. Thus these three types of ligands affect GABA-dependent gating of the chloride channel with positive, negative, or zero intrinsic efficacy. Compounds have re-
cently been synthesized which possess both agonist and antagonistic properties. Such partial agonists may be even safer and more selective as anxiolytic drugs.

Phylogenetic studies reveal that benzodiazepine binding sites are present in vertebrate but not invertebrate species, suggesting a late evolutionary appearance (85). This indicates an important physiological role for the receptor and points to the possibility of an endogenous ligand for this site. Clearly, the identification of such a compound would provide important information with regard to the biological mechanisms regulating anxiety, seizure threshold and sleep. While numerous investigators have attempted to isolate such a substance, no compound is as yet universally accepted as the endogenous benzodiazepine receptor ligand. Interest is presently focused on a peptide, diazepam binding inhibitor (DBI), which has been extracted from brain and has properties similar to those of inverse agonists (1,24,49). DBI has an affinity constant of 1 nM for the benzodiazepine binding site. Since DBI is present in only some GABA neurons it may serve as a ligand for only a select group of benzodiazepine receptors. Moreover, DBI is found in non-GABAergic neurons, suggesting that it may also serve some function unrelated to the benzodiazepine site (1).

While the identification of a specific binding site for benzodiazepines strongly suggests the presence of an endogenous ligand, it does not prove its existence. Attempts to demonstrate the physiological effects of an endogenous ligand by inhibiting its receptor interaction with the benzodiazepine anta-
agonist RO 15-1788 have been inconclusive thus far.

Studies have uncovered a variety of benzodiazepine agonists which differ in their pharmacological profiles (53). Some possible explanations for these differences include the presence of benzodiazepine receptor subclasses, only some of which mediate anxiolytic actions while others are important for sedative and muscle relaxant effects (62,68,74). However, there is no direct evidence supporting the existence of molecularly distinct benzodiazepine receptors, making it conceivable that the heterogeneity suggested from binding studies reflects different conformations of a single benzodiazepine site (57,80). On the other hand, differences in pharmacological profiles may be due to variations in intrinsic efficacy and in the extent of receptor reserve among neurons (55). For example, it is possible that neurons associated with anxiety or epileptic activity have a higher receptor reserve than those controlling alertness or muscle tone. Full agonists would produce a maximal affect when all receptors are activated on cells having no receptor reserve, whereas partial agonists would display only limited activity. In contrast, partial agonists may yield a maximal response in those cells possessing a significant amount of receptor reserve.

Although many questions remain about the molecular structure of the GABA receptor complex, there is sufficient information to propose a working model (Figure 2). Receptor purification experiments indicate that the GABA/benzodiazepine moiety is a glycoprotein containing two subunits, with the α-subunit having a molecular weight of 50 kd and the β-subunit 55 kd. A tetrameric αβ2 arrangement is suggested by comparing the mole-
cular weights of the subunits with the native receptor (76, 80, 95, 96). The α/β structure accommodates not only the binding sites for GABA and benzodiazepines, but also the TBPS binding site (96) which is associated with the chloride channel (99) (Figure 1). Electrophysiological studies suggest the presence of two GABA recognition sites for each GABA receptor-associated chloride channel (91).

Little is known about the precise location of the various ligand binding sites on the GABA receptor domain. Photoaffinity labeling suggests that the binding sites for the benzodiazepines and GABA are present on the α-subunit, although they may also be located on the β-subunit in a state that is not generally labeled (76, 95). The exact location of the binding sites for barbiturates and picrotoxin is unknown. It is conceivable that these may be present on subunit interfaces.

While the present model is consistent with the majority of experimental data, recent findings indicate that its design will have to be modified. For instance, it appears that an additional protein is present in certain purified receptor preparations (93). Furthermore, the target size of the radiation-inactivated TBPS binding site appears to be exceptionally large as compared to the size of the GABA/benzodiazepine complex (84). This might be explained by the presence of an additional subunit of 62-80 kD (γ-subunit) in the receptor complex (80). More precise information on the structure, synthesis and assembly of the receptor will be forthcoming with the isolation of GABA receptor genes. This development will also provide DNA probes to identify those
cells which express GABA receptors.

The intimate association between the benzodiazepine binding site and GABA receptors suggests that disorders such as epilepsy, anxiety, and insomnia might result from a deficit in GABA receptor function, or in the activity of selected GABAergic neurons. Indeed it has been suggested that GABAergic transmission is altered in the vicinity of epileptic foci, suggesting that inhibitory influences may be insufficient to prevent the generalized spread of paroxysmal discharges (16). It is also possible that a defect in the GABAergic control of certain excitatory stimuli might contribute to anxiety, an hypothesis based on the finding that inverse agonists induce anxiety in human subjects (31). As for insomnia, it has been found that sleep latency is prolonged by the benzodiazepine inverse agonists and is diminished by benzodiazepine agonists, suggesting an involvement of GABAergic systems in the etiology of some sleep disorders. More definitive information with regard to these issues may soon be obtained with positron emission tomography using benzodiazepine ligands as the emitting isotopes (33, 92).

GABA RECEPTORS

β-4-Chlorophenyl GABA (baclofen) was designed as a centrally-active GABA receptor agonist (8). However, the electrophysiological response to baclofen is resistant to blockade by bicuculline and picrotoxin, suggesting that its effects are mediated by an action other than direct activation of GABA receptors (18). Recently it has been found that baclofen induces some
responses that are mimicked by GABA, indicating that it may be a selective agonist for a receptor subgroup (GABA receptors) that are resistant to the classical GABA receptor antagonists (11,65).

Ligand binding assays suggest that the GABA binding site is associated with divalent cations, in particular calcium (41). Functional assays indicate that the GABA site may be important for regulating neurotransmitter release and may be associated with second messenger production in brain (9,38,42,44). Like the GABA binding site, GABA receptors are widely distributed throughout the central nervous system (63). Unlike GABA receptors, the GABA site is not associated with chloride ion channels or benzodiazepines (9). A major hindrance to the characterization of GABA receptors is the absence of potent and selective antagonists for this site. Indeed, the existence of GABA receptors will remain a matter of dispute until selective antagonists are found.

One action attributed to GABA receptors is a regulatory role with respect to second messenger responses in brain (Figure 3) (58,65,64,110). Although neither baclofen nor GABA have any direct effect on cyclic AMP production themselves, both agents amplify the production of this second messenger when brain tissue is exposed to a neurotransmitter that directly stimulates the accumulation of this cyclic nucleotide. For example, a saturating concentration of isoproterenol causes an 8-fold increase in cyclic AMP production in rat brain cerebral cortical slices. In the presence of baclofen or GABA, isoproterenol-stimulated cyclic
AMP accumulation is over 20-fold higher than basal levels, indicating that GABA receptor activation amplifies the second messenger response to the β-adrenergic agonist. Similar results were obtained when cyclic AMP production was activated by norepinephrine, vasoactive intestinal peptide or adenosine (65). Importantly, selective GABA receptor agonists such as isoguvacine and THIP are inactive in this regard, and the response to baclofen is insensitive to blockade by bicuculline or picrotoxin (65,64). These findings suggest that the second messenger response to baclofen is mediated by a GABA receptor distinct from GABA sites. Thus GABA, through an action at GABA receptors, may serve to modulate the receptor responses to a variety of neurotransmitters in brain.

Experiments conducted to define the biochemical properties of GABA<sub>B</sub> receptors indicate that the amplification phenomenon is totally dependent upon the presence of extracellular calcium ion (32,64). Moreover, it appears that stimulation of GABA<sub>B</sub> receptors may result in the activation of phospholipase A<sub>2</sub>, a calcium-dependent enzyme that catalyzes the conversion of phospholipids to arachidonic acid (Figure 3). While arachidonic acid is rapidly converted to prostaglandins and a variety of hydroperoxy derivatives, these metabolites do not seem to contribute to the augmentating response (32). This suggests that arachidonate, or some other fatty acid, may mediate the GABA receptor-induced augmentation of cyclic AMP production. In this regard it is interesting that arachidonic acid is capable of activating C kinase (82), an enzyme known to phosphorylate a variety of intracellular proteins. In platelets C kinase phosphorylates a GTP-
binding protein (G↓) known to inhibit adenylate cyclase activity (67). Therefore it is conceivable that by stimulating the formation of arachidonic acid, GABA receptor activation reduces the influence of G↓ on adenylate cyclase, thereby enhancing the responsiveness of the neurotransmitter receptor-coupled cyclic AMP generating system. This model must be considered highly speculative, however, until more direct evidence is provided that phospholipase A is activated by GABA agonists and that G↓ is phosphorylated following exposure to baclofen.

The finding that GABA may act as a neuromodulator has significant implications with regard to psychotherapeutics. For example, the monoamine theory of depression suggests that this disorder is secondary to an alteration in brain noradrenergic and serotonergic transmission (48). Since the transmitters for both systems are associated with cyclic AMP production in brain it is conceivable that some forms of depression may be due to a GABA receptor dysfunction that diminishes the responsiveness of the norepinephrine and serotonin receptors. In this case a GABA B agonist may be beneficial in the treatment of affective illness either alone or in combination with standard medications. A recent study has indicated that co-administration of baclofen with imipramine facilitates the appearance of a neurochemical response thought to be related to the therapeutic efficacy of antidepressants (42).

Schizophrenia appears to be associated with excessive dopaminergic tone in critical areas of the brain (87). Inasmuch as one type of dopamine receptor (D-1) is associated with adenylate
cyclase, it is conceivable that some symptoms of schizophrenia may be due to enhanced GABA receptor activity. That is, an overactive GABA receptor system may amplify dopaminergic responses even though dopamine turnover may be unaltered. This makes it conceivable that GABA receptor antagonists might have antipsychotic potential. While highly speculative, such theories are consistent with the present information and serve to illustrate why continued research on GABA receptors may lead to the development of novel therapeutic agents.

CONCLUSIONS

Early studies on the chemical nature of synaptic transmission concentrated on presynaptic events since there were few biochemical methods for studying postsynaptic mechanisms. Because of this emphasis a great deal was learned about the actions of drugs that influence the concentration or turnover of neurotransmitter substances. For example, psychopharmacological agents were found to modify the storage (reserpine), release (amphetamine), metabolism (pargyline), or reuptake (imipramine) of monoamines. It has become possible in recent years to examine directly the interaction of drugs with synaptic receptor sites (111). This has led to the discovery that some psychoactive drugs act by directly stimulating (lysergic acid diethylamide) or inhibiting (haloperidol) transmitter receptors (4,27). Moreover, it has been found that direct stimulation or inhibition of receptor recognition sites are not the only ways to modify receptor function (61,65,101). Thus it appears that transmitter receptors are macromolecular complexes containing a family of interacting
sites, each of which may be manipulated for therapeutic gain.

This concept developed in part as a consequence of research on GABA receptors. One of the initial breakthroughs came with the discovery that certain GABA receptor antagonists, such as picrotoxin, selectively alter receptor function by acting on a component other than the GABA recognition site. Of special interest to psychopharmacologists was the discovery that benzodiazepines facilitate GABAergic transmission by attaching to a receptor component physically distinct from the GABA binding site. This demonstrated that by acting upon receptor components separate from the recognition site drugs can exert subtle effects on neurotransmitter systems.

Work during the past decade has revealed that the benzodiazepine component of the GABA receptor has characteristics that distinguish it from classical neurotransmitter receptor recognition sites. Thus, not only have substances been found that activate (diazepam and chlorodiazopoxide) and inhibit (RO 15-1788) this site, but there are also agents evoking a response totally opposite from diazepam (inverse agonists).

The concept that neurotransmitter receptors may be subtly manipulated by drugs was reinforced by the discovery that GABA, through an action at GABA receptors, may act as a neuromodulator rather than a neurotransmitter. In this case the GABA_B binding site appears to be affiliated with neurotransmitter receptors that are directly coupled to the cyclic AMP generating system. Thus, receptor responses are a function not only of the amount of transmitter released but also of receptor responsive-
ness which appears to be under the control of modulating substances.

Such findings have implications with regard to defining the biological abnormalities associated with mental illness. Because it has been difficult to identify neurochemical lesions associated with most psychiatric diseases, it appears that neurotransmitter synthesis, storage and release may not be dramatically altered in these conditions. The discovery that receptor activity may be continuously regulated by neuromodulators and receptor site-associated components make it conceivable that some forms of mental illness are secondary to an alteration in these regulatory systems. Thus, studies on GABA neurotransmission have not only yielded insights with regard to the characteristics of this receptor, they have also provided new perspectives with regard to receptor mechanisms, the etiology of psychiatric illness, and the development of novel therapeutic agents.

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FIGURE LEGENDS

FIG. 1 Schematic representation of the components associated with GABA receptors. Agents interacting at each site are listed above the individual components.

FIG. 2 Structural model of the GABA/benzodiazepine receptor/chloride channel complex. The α and β symbols indicate subunits differentiated on the basis of their molecular weights (50 and 55 kd, respectively). MAB I and MAB II are distinct epitopes recognized by subunit specific monoclonal antibodies. Photolabeling studies indicate that the GABA and benzodiazepine (Benzo) binding sites are located on the MAB II subunits, although the precise location of each binding domain remains unknown.

FIG. 3 Schematic representation of the components proposed for the GABA receptor system. In this model the GABA receptor is in the vicinity of recognition sites for cyclic AMP-coupled transmitter systems. G_i and G_s represent inhibitory and stimulatory guanine nucleotide binding proteins, respectively. Agonists for the two receptor recognition sites are listed above each component.
<table>
<thead>
<tr>
<th>AGONISTS</th>
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<th>ANTAGONISTS</th>
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<tbody>
<tr>
<td>DIAZEPAM</td>
<td>MUSCIMOL</td>
<td>Picrotoxin</td>
</tr>
<tr>
<td>CHLORDIAZEPoxide</td>
<td>THIP</td>
<td>TBPS</td>
</tr>
<tr>
<td>FLUNITRAZEPAM</td>
<td>ISOGUVACINE</td>
<td>TBOB</td>
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<tr>
<td>ANTAGONISTS</td>
<td></td>
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<tr>
<td>RO 15-1788</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CGS- 8216</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INVERSE AGONISTS</td>
<td>ANTAGONISTS</td>
<td></td>
</tr>
<tr>
<td>β-CARBOLINE CARBOXYLATE ETHYL ESTER</td>
<td>BICUCULLINE</td>
<td>SECURININE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SR 95103</td>
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**FIG. 1**
CYCLIC AMP-COUPLED RECEPTOR AGONISTS

- NOREPINEPHRINE
- DOPAMINE
- VIP
- ADENOSINE

GABA\textsubscript{B} RECEPTOR AGONISTS

- BACLOFEN
- KOJIC AMINE

\begin{tikzpicture}

% TikZ code for the diagram

\end{tikzpicture}

Ca\textsuperscript{++}

FIG. 3
RECEPTOR-MEDIATED MODULATION OF NEUROTRANSMITTER-STIMULATED CYCLIC AMP ACCUMULATION IN RAT BRAIN SLICES

S. J. ENNA\(^1\) AND E. W. KARBON\(^2\)

\(^1\)Nova Pharmaceutical Corporation, 5210 Eastern Avenue, Baltimore, Maryland 21224, and \(^2\)Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510.

INTRODUCTION

Neurotransmitter receptors regulate cellular activity in at least two ways; through a direct coupling to an ion channel, or through an association with a second messenger system (1). In the latter case, activation of the receptor recognition site initiates a series of biochemical events resulting in the stimulation or inhibition of an enzyme that catalyzes the formation of an intracellular second messenger such as cyclic AMP or diacylglycerol. These substances in turn promote protein phosphorylation by stimulating kinase activity (2,3).

One characteristic of receptor function that has received intense scrutiny in recent years is the manner in which the sensitivity of these sites is regulated (4,5). Thus, prolonged activation or blockade of receptor recognition sites can lead to a decrease or increase, respectively, in their density. Such a change is thought to be important for maintaining synaptic homeostasis. Transmitter receptors are subject to short-term regulation as well. For example, the benzodiazepines, a class of central nervous system depressants, influence chloride ion flux by altering the sensitivity of \(\gamma\)-aminobutyric acid (GABA) receptor sites, an effect that occurs immediately upon exposure to these drugs. Such discoveries have reinforced the notion
that synaptic activity is under the continuous influence of regulators that maintain a proper balance between the degree of receptor occupancy and the effector response.

Given the number of components associated with receptor-mediated second messenger systems, it is not surprising that these signal transduction pathways may be influenced by neuromodulatory substances. With regard to the adenylate cyclase system, activation of certain receptors, e.g., adrenergic, leads to a coupling between the receptor and a guanine nucleotide binding protein, Ns. The N protein-GTP complex stimulates the catalytic unit of adenylate cyclase, facilitating the conversion of ATP to cyclic AMP. Intracellular levels of cyclic AMP are regulated by the amount and activity of adenylate cyclase, as well as by phosphodiesterases that convert cyclic AMP to the corresponding 5'-nucleotide. The interactive nature of this system makes possible a rapid modification in receptor function through an alteration in any one of these components.

Among the first to describe a receptor-mediated, indirect influence on cyclic AMP accumulation were Daly and his colleagues (1,2). These investigators found that although α-adrenergic receptor agonists have little influence on second messenger accumulation in brain tissue, they greatly amplify the cyclic AMP response observed during exposure to substances, e.g., isoproterenol, that directly stimulate adenylate cyclase. It was concluded that the brain α-adrenergic receptor system, while not directly affiliated with adenylate cyclase, can modify the rate or amount of cyclic AMP produced by a neurotransmitter that directly activates the enzyme. This implies that certain endogenous agents modulate neurotransmitter receptor responses in brain. More recent work has suggested that one of these is GABA (9,10). Thus, GABA_B, but not GABA_A, receptor agonists augment cyclic AMP accumulation in brain slices in a manner similar to that reported for α-adrenergic receptor substances (9,10). This suggests that GABA, through an interaction with a subclass of GABA receptors, regulates the receptor responses elicited by a variety of neurotransmitter agents.

The present report is designed to highlight data supporting the concept of a receptor-mediated augmentation of second messenger responses in brain. Particular emphasis is placed on findings related to the biochemical properties of this regulatory phenomenon, and on the implication of these results with respect to the design of new therapeutic agents.

**AUGMENTATION OF CYCLIC AMP RESPONSES**

It has been estimated that up to 40% of central nervous system neurons contain GABA. Given the high concentration and widespread distribution of
GABA, it seems likely that the brain contains several pharmacologically distinct receptors for this substance. The best characterized of these is that associated with chloride ion channels (11). These receptors, classified as GABA_A, are blocked by bicuculline and, in some cases, are associated with benzodiazepine binding sites. Studies with 6-p-chlorophenyl GABA (baclofen), a GABA_A antagonist, suggest a second class of GABA receptors, the GABA_B site (12). These receptors are not blocked by bicuculline. In fact, no selective antagonists have yet been discovered for this site. Nevertheless, a variety of bicuculline-resistant responses to baclofen and GABA have been identified, supporting the existence of a GABA receptor population that differs from the GABA_A site. Studies aimed at defining the effector mechanism associated with GABA_B receptors included experiments to determine whether the GABA_B system is associated with second messenger production. While it has been found that GABA agonists inhibit adenylate cyclase in rat brain homogenates (13), neither GABA nor baclofen directly modifies cyclic AMP accumulation in brain slices (10), although they augment the cyclic AMP response observed in the presence of isoproterenol, a β-adrenergic receptor agonist (Table 1.). Thus, in the presence of baclofen, the cyclic AMP response to a saturating concentration of the β-adrenergic agonist is increased approximately 3-fold. This augmentation is concentration-dependent, with the EC50 for baclofen being approximately 6 μM. The increased response obtained with the isoproterenol–baclofen combination is not due to a baclofen-mediated inhibition of phosphodiesterase, nor is it the result of a baclofen-induced increase in β-adrenergic receptor affinity or number (10). Moreover, the baclofen response is mimicked by GABA itself, suggesting that it is mediated by a GABA receptor system. Furthermore, the augmentation is not restricted to β-adrenergic receptors, but is observed with many substances known to stimulate adenylate cyclase in brain, including vasoactive intestinal peptide (VIP), adenosine and histamine (10). These findings suggest that GABA, through an action at GABA_B receptors, rather than mediates, cyclic AMP accumulation in brain.

The results with baclofen are remarkably similar to those obtained for β-adrenergic agonists (8, 14, 15). Thus, when rats were incubated with 6-fluoronorepinephrine, a non-selective β-adrenergic agonist, no change in cyclic AMP accumulation was noted. However, co-incubation of 6-fluoronorepinephrine with isoproterenol increased the amount of second messenger produced by the β-adrenergic agonist. In this case, a saturating concentration of 6-fluoronorepinephrine doubled the response.
GABA\textsubscript{B} agonists, \(\alpha\)-adrenergic agonists augment the cyclic AMP response to numerous neurotransmitters (14). Pharmacological studies suggest that the \(\alpha\)-adrenergic receptor-mediated augmentation of second messenger accumulation has characteristics of both \(\alpha_1\) and \(\alpha_2\)-adrenergic receptor systems.

Table 1. The influence of baclofen, 6-fluoronorepinephrine, and phorbol esters on basal and isoproterenol-stimulated cyclic AMP accumulation in rat brain cerebral cortical slices. Tissue was prelabeled with \(^3\)H-adenine and then incubated with baclofen (50 \(\mu\)M) 6-fluoronorepinephrine (10 \(\mu\)M) or one of the phorbol esters (10 \(\mu\)M) alone or in combination with isoproterenol (10 \(\mu\)M), after which \(^3\)H-cyclic AMP was isolated and quantified. Each value represents the mean \(\pm\) s.e.m. of 4-6 separate experiments, each of which was analysed in duplicate. Adapted from (10, 14, 19).

* \(P < 0.05\) compared to isoproterenol alone (two-tailed t-test).

<table>
<thead>
<tr>
<th>Incubation Condition</th>
<th>Basal</th>
<th>With Isoproterenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.06 (\pm) 0.005</td>
<td>0.38 (\pm) 0.03</td>
</tr>
<tr>
<td>Baclofen</td>
<td>0.11 (\pm) 0.02</td>
<td>1.14 (\pm) 0.09*</td>
</tr>
<tr>
<td>6-Fluoronorepinephrine</td>
<td>0.07 (\pm) 0.005</td>
<td>0.77 (\pm) 0.04*</td>
</tr>
<tr>
<td>4-6-Phorbol 12,13-Dibutyrate</td>
<td>0.06 (\pm) 0.004</td>
<td>1.20 (\pm) 0.07*</td>
</tr>
<tr>
<td>4-(\alpha)-Phorbol</td>
<td>0.05 (\pm) 0.005</td>
<td>0.36 (\pm) 0.03</td>
</tr>
</tbody>
</table>

(14, 15). It is interesting that, as with GABA\textsubscript{B} receptor agonists, \(\alpha_2\)-adrenergic agonists have been shown to inhibit adenylate cyclase activity under certain conditions (16).

POSSIBLE INVOLVEMENT OF PHOSPHOLIPASE \(A_2\) AND PROTEIN KINASE C

Experiments aimed at defining the mechanism whereby GABA\textsubscript{B} and \(\alpha\)-adrenergic agonists augment cyclic AMP accumulation have revealed a strict calcium dependency for this phenomenon. Thus, the calcium chelator EGTA has no effect on isoproterenol-stimulated cyclic AMP accumulation in rat brain cerebral cortical slices at concentrations up to 2.5 \(\mu\)M (Table 2). However, this concentration of EGTA reduces the cyclic AMP response observed in the presence of
baclofen and isoproterenol, with the maximal reduction being to a level identical to that found with isoproterenol alone, suggesting that EGTA eliminates the baclofen-mediated augmentation. Likewise, EGTA attenuates the cyclic AMP response to norepinephrine to the level found with isoproterenol alone, indicating a selective elimination of the α-adrenergic receptor response (Table 2).

Table 2. The effect of EGTA and quinacrine on receptor-stimulated cyclic AMP accumulation in rat brain cerebral cortical slices. The tissue was incubated with norepinephrine (100 μM) or isoproterenol (10 μM) alone, or with isoproterenol and baclofen (50 μM) after prelabeling with 3H-adenine. In some cases the incubations were conducted in the presence of 2.5 μM EGTA or 250 μM quinacrine. Following these exposures, 3H-cyclic AMP was isolated and quantified. Each value represents the mean ± s.e.m. of 3-5 separate experiments, each of which was conducted in duplicate. Adapted from (15).

*P < 0.05 compared to corresponding control (two-tailed t-test).

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Control</th>
<th>+ EGTA</th>
<th>+ Quinacrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoproterenol</td>
<td>0.54 ± 0.04</td>
<td>0.58 ± 0.05</td>
<td>0.49 ± 0.05</td>
</tr>
<tr>
<td>Baclofen-Isoproterenol</td>
<td>1.95 ± 0.24</td>
<td>0.54 ± 0.08*</td>
<td>1.02 ± 0.08*</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>1.50 ± 0.12</td>
<td>0.52 ± 0.07*</td>
<td>0.50 ± 0.05*</td>
</tr>
</tbody>
</table>

These data have been taken as evidence that the augmenting response is associated with a calcium-dependent enzyme. Given the suggestion that cyclic AMP production may be influenced by prostaglandins (17), it seems possible that phospholipase A2 (PLA2), a calcium-dependent enzyme that catalyzes the formation of arachidonic acid, the precursor of prostaglandins, may participate in the response. To test this, the α-adrenergic and GABA B-mediated augmentation of second messenger production was examined in the presence of quinacrine, a non-selective inhibitor of PLA2 (15). As with EGTA, quinacrine (250 μM) completely eliminated the α-adrenergic component of norepinephrine-stimulated cyclic AMP accumulation in rat brain slices (Table 2). Moreover, quinacrine greatly reduced the baclofen-mediated augmentation
of cyclic AMP accumulation (Table 2). This effect of quinacrine is concentration-dependent, displaying an EC₅₀ of approximately 120 μM.

Because of the non-selective nature of quinacrine, it is possible that its effect on the augmenting response is due to some action unrelated to PLA₂. However, quinacrine alone does not influence isoproterenol-stimulated cyclic AMP accumulation, suggesting that it does not directly inhibit adenylate cyclase. Moreover, chronic administration of glucocorticoids or ACTH causes a reduction in the GABA₂ - and α-adrenergic-mediated augmentation (15). Inasmuch as glucocorticoids stimulate the production of endogenous inhibitors of PLA₂, this finding supports the notion that PLA₂ may be an important mediator of the augmenting response to GABA₂ and α-adrenergic agonists. Interestingly, substances that inhibit the metabolism of arachidonic acid do not selectively modify the augmenting response to GABA₂ and α-adrenergic agonists (17). This suggests that arachidonic acid itself, or perhaps lysophospholipid, mediates the actions of baclofen and α-adrenergic agonists on cyclic AMP accumulation.

Tumor promoting phorbol esters, such as 4-β-phorbol 12,13-dibutyrate (PDBu) are also capable of augmenting second messenger responses in brain slices (18, 19). As with baclofen, PDBu has no effect on cyclic AMP accumulation itself, while greatly amplifying the production of this second messenger in the presence of isoproterenol or other agents that directly stimulate adenylate cyclase (Table 1). At a concentration of 10 μM, PDBu increases isoproterenol-stimulated cyclic AMP accumulation almost 4-fold in rat brain cerebral corical slices. The similarity between the responses to PDBu, baclofen and α-adrenergic agonists suggest they may act by a similar mechanism.

A prominent action of PDBu is stimulation of protein kinase C, a calcium-dependent enzyme that catalyzes the phosphorylation of a variety of proteins (3). Importantly, phorbol esters incapable of stimulating protein kinase C, such as 4α-phorbol, are incapable of augmenting cyclic AMP accumulation (Table 1). This makes it appear that stimulation of protein kinase C is a critical factor in the cyclic AMP augmenting response to these substances. Protein kinase C is stimulated in vivo by diacylglycerol, a second messenger produced by the action of phospholipase C, a neurotransmitter receptor-coupled enzyme (20). While neither GABA₂ nor α₂-adrenergic agonists stimulate phospholipase C (15), a number of phospholipids, including arachidonic acid, do (21,22). Therefore it is possible that the arachidonic acid formed by the action of these substances could stimulate protein kinase C, mimicking the effect of tumor-promoting phorbol esters. It is conceivable, however, that although the augmenting effect of phorbol esters, GABA₂ and α-adrenergic agonists are similar, they act by different mechanisms. In fact, it has been shown that prolonged exposure to phorbol esters causes a
down-regulation of protein kinase C activity in rat brain slices and an attenuation of their cyclic AMP augmenting response (19, 23). However, this treatment had no effect on GABA_B or α-adrenergic receptor-mediated augmentation of cyclic AMP accumulation, implying that protein kinase C may not contribute to the action of these agents. In this regard, it would be useful to identify those proteins associated with the cyclic AMP system that are phosphorylated during activation of kinase C to determine whether baclofen and α-adrenergic agonists influence similar substrates. In platelets it has been found that stimulation of protein kinase C causes the phosphorylation of the alpha-subunit of the inhibitory guanine nucleotide binding protein, Ni (24). Thus it is possible that the phosphorylation and subsequent inactivation of inhibitory influences could account for the increase in the responsiveness of the adenylate cyclase system noted with baclofen, α-adrenergic agonists and PDBu. Taken together, these findings have yielded a series of testable hypotheses that can be explored to define the mechanism(s) whereby endogenous substances, through an action at brain receptor sites, modulate second messenger systems.

CONCLUSION

One of the more precise ways to influence central nervous system function is to administer drugs that interact with a particular group of neurotransmitter receptors. However, direct inhibition or stimulation of these sites may not be the most effective way to overcome a neurochemical imbalance. Given the likelihood that a certain level of synaptic activity is necessary for optimal functioning, complete receptor blockade with antagonists, or a direct stimulation with agonists, is unlikely to yield the appropriate balance necessary for establishing normal activity. A more desirable strategy may be to develop drugs that manipulate synaptic function in a more subtle manner, such as occurs with endogenous modulatory substances.

Support for this hypothesis is provided by the findings with benzodiazepines, one of the safest and most effective classes of central nervous system drugs. The unique clinical profile of these agents appears due to their ability to facilitate GABA receptor responses rather than to directly activate this system. By acting in this way, the benzodiazepines do not stimulate the transmitter receptor beyond its normal limits, yielding a more modest, although more therapeutically useful, response. Given such findings, it is important to identify those substances in brain that are capable of regulating neurotransmitter receptor responses in order to design new therapeutic agents that can manipulate these systems.

Among the regulators identified are GABA and α-adrenergic agonists. As
opposed to classical neurotransmitters, these substances alone have no direct
effect on cyclic AMP accumulation in brain slices, but rather augment the
production of this second messenger that occurs in the presence of other
agents such as β-adrenergic agonists, adenosine, and VIP. Assuming that a
similar response occurs in vivo, these data suggest that GABA and endogeneous
agonists for the α-adrenergic system may act as neuromodulators rather than
neurotransmitters. While the dysfunction of such a neuromodulatory system may
cause only a modest change in brain neurochemistry, it could bring about a
dramatic alteration in behavior. Therefore it is conceivable that one of the
difficulties associated with identifying the biological abnormalities
responsible for major psychiatric illnesses may be because these disorders are
related to an absence or overabundance of neuromodulatory activity rather than
to a dramatic change in neurotransmitter function. Even if mental illness is
unrelated to a change in neuromodulation, the manipulation of such systems
could be of therapeutic benefit. Indeed it has been reported that baclofen
facilitates the antidepressant-induced change in brain neurochemistry that is
thought to be associated with the clinical response to these agents (25). A
better understanding of the biochemical mechanisms associated with
neuromodulator receptor function could lead to the development of drugs that
can alter brain neurotransmitter activity by either directly modifying the
modulator site or its association with the neurotransmitter receptor. Besides
being therapeutically useful, such agents could aid in defining the biological
abnormalities associated with neuropsychiatric illness.

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KEY WORDS: Cycle AMP, γ - Aminobutyric Acid, Neuromodulators, α - Adrenergic Receptors, Phorbol Esters, Protein Kinase C.
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