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TRICYCLIC ANTIDEPRESSANTS AND IMIDAZOLINES AS INHIBITORS OF THE ALPHA-ADRENERGIC RECEPTOR MEDIATED STIMULATION OF PHOSPHATIDYLINOSITOL TURNOVER IN RAT PINEAL GLAND

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Abstract—In intact rat pineal glands *in vitro*, norepinephrine stimulates the incorporation of ^{32}P into phosphatidylinositol through post-synaptic alpha-adrenergic receptors. This response is inhibited by tricyclic antidepressants and imidazolines. Both amitriptyline and oxymetazoline displace the dose–response curve for norepinephrine to the right in a parallel manner indicative of a competitive mode of inhibition by these two classes of drugs.

Phospholipids do not function solely as relatively inert structural components of cell membranes, in which the more metabolically active receptor proteins reside. An accumulation of recent evidence suggests that within cell membranes a dynamic interaction exists between phospholipids and receptor proteins. For example, dopaminergic, opiate, muscarinic, cholinergic and adrenergic receptor activities can be modulated by altering their membrane phospholipid environment [1–5]. Conversely, activation of a variety of receptors enhances the turnover of certain phospholipids, of which phosphatidylinositol is the most prominent (for review see Michell [6]).

In earlier studies with the rat pineal gland, we demonstrated the dose–response relationship between norepinephrine activation of the alpha-adrenergic receptor and the enhanced incorporation of ^{32}P into acidic phospholipids, namely phosphatidylinositol, phosphatidylglycerol, and phosphatidic acid [7]. The dramatic increase in ^{32}P incorporation into phosphatidylinositol due to stimulation by norepinephrine has been called the phosphoinositide effect, which in the pineal gland, as in other tissues (for review see Jones and Michell [8]), is specific for alpha-receptor activation [9]. In addition to norepinephrine, methoxamine and phenylephrine, both full agonists at post-synaptic alpha-adrenergic receptors, are able to elicit the phosphoinositide effect [10]. It can also be inhibited by classical alpha-antagonists such as phentolamine and phenoxybenzamine, while sotalol, a pure beta-adrenergic antagonist, has no effect. We have also shown that the phosphoinositide effect is not altered after destruction of presynaptic adrenergic nerve terminals by chronic 6-hydroxydopamine pretreatment [11]. Furthermore, we demonstrated recently the phosphoinositide effect in dispersed pineal cells (unpublished results). These results suggest a post-synaptic locus for the alpha-receptor response.

Through the use of binding studies employing such radioligands as [^{3}H]WB4101 and [^{3}H]clonidine, investigators have been able recently to characterize the alpha-receptor more clearly [12, 13]. Yet, because of the lack of well-defined biochemical mechanisms associated with activation of alpha-adrenergic receptors (for review see Kunos [14]), binding studies by themselves necessarily yield limited information. Therefore, we have employed the phosphoinositide effect as a biochemical response to evaluate the activities of two classes of compounds, tricyclic antidepressants and imidazolines, both of which have been shown by radioligand binding [12] and physiological responses [15, 16] to possess either full antagonist or partial agonist actions on alpha-adrenergic receptors. A preliminary report of these findings has been presented [17].

METHODS AND MATERIALS

Materials

Pharmacological agents. (−)-Norepinephrine bitartrate was obtained from the Regis Chemical Co., Morton Grove, IL 60053; clonidine HCl from Boehringer Ingelheim, Ltd., Elmsford, NY 10523; oxymetazoline HCl from the Schering Corp., Kenilworth, NJ 07033; naphazoline HCl, xylometazoline HCl and tolazoline HCl from the Ciba–Geigy Corp., Summit, NJ 07901; cocaine HCl from Mallinckrodt Inc., St. Louis, MO 63134; and tricyclic antidepressants from Merck, Sharpe & Dohme, West Point, PA 19486.

Other materials. Modified Puck's N-16 medium was purchased from the Grand Island Biological Co., Grand Island, NY 14072; and silica gel H thin-layer plates were obtained from Analtech, Inc., Newark, DE 19711. Kodak No-Screen 54 NT X-ray film was used for radioautography. $^{32}PH_3PO_4$ was purchased from New England Nuclear, Boston, MA 02118.

Methods

Female rats (Charles River Breeding Laboratories, Wilmington, MA 01887), weighing 150–200 g, were decapitated and the pineal glands removed and freed of extraneous tissue. Individual pineal glands were incubated with shaking for 60 min at 37° in glass tubes (10 × 75 mm) containing 0.1 ml modified Puck's N-16 medium (pH 7.4) to which 10 μCi $^{32}P_i$ (final specific activity 70 μCi/μmole) plus drugs, when indicated, had been added. Incubations were terminated by the addi-

tion of 2 ml of ice-cold saline. The pineal glands were quickly removed, washed two additional times in 2 ml of ice-cold saline and transferred to 3.5 ml $CHCl_3$–CH_3OH (2:1, v/v) containing 5% H_2O plus carrier rat brain lipid extract prepared according to the procedure of Folch *et al.* [18]. After standing a minimum of 1 hr at room temperature, 0.2 vol. of 0.01 N HCl was added and the mixture was shaken. After phase separation, the upper phase and the extracted gland were discarded and the lower phase was washed two additional times with $CHCl_3$–CH_3OH–0.01 N HCl (3:48:47, v/v). One drop of concentrated NH_4OH was added to the washed lower phase which was then dried under a stream of nitrogen, redissolved in a few drops of $CHCl_3$–CH_3OH–H_2O (75:25:2 by volume) and transferred quantitatively to 20 × 20 cm silica gel H thin-layer plates.

In some experiments, two-dimensional chromatography was employed to separate individual phospholipids. The plates were developed in the first dimension with $CHCl_3$–CH_3OH–CH_3COOH–H_2O (52:20:7:3 by volume), air dried and washed with acetone as described previously [7]. The solvent employed for the second dimension was $CHCl_3$–CH_3OH–40%CH_3NH_2–H_2O (65:31:5:5 by volume). The plates were then radioautographed for 48–72 hr to visualize radioactive areas corresponding to phosphatidylinositol, phosphatidylcholine, phosphatidylglycerol, phosphatidylethanolamine and phosphatidic acid which were scraped and counted. In other experiments, thin-layer plates were run only in one dimension with $CHCl_3$–CH_3OH–CH_3COOH–H_2O (25:15:4:2 by volume) when it was desired to isolate only phosphatidylinositol and phosphatidylcholine. The plates were exposed to I_2 vapors to locate these lipids. Although phosphatidylserine co-chromatographed with phosphatidylinositol in one- and two-dimensional chromatography, phosphatidylserine incorporated less than 1 per cent of the label compared to over 40 per cent for phosphatidylinositol [7]. Thus, activity associated with phosphatidylserine was negligible.

Since no increase in phosphatidylcholine radioactivity above control values is observed during norepinephrine stimulation, phosphatidylinositol radioactivity was normalized for each pineal gland by expressing ^{32}P incorporation into phosphatidylinositol as the ratio of incorporation into phosphatidylinositol to incorporation into phosphatidylcholine, thus avoiding errors due to variations in ^{32}P uptake in different glands. This is abbreviated as phosphatidylinositol/phosphatidylcholine in the figures.

RESULTS

Inhibitory effects of tricyclic antidepressants

All of the tricyclics tested at 1.5 μM, with the exception of protriptyline, inhibited the enhanced incorporation of ^{32}P into phosphatidylinositol elicited by 10 μM norepinephrine (Fig. 1). The secondary amine tricyclic, desipramine, reduced the incorporation ratio achieved with norepinephrine alone from 6.84 ± 0.39 to 5.13 ± 0.32, while protriptyline was not effective. The tertiary amine tricyclics, amitriptyline, doxepin and imipramine, tended to be more potent inhibitors than the secondary amines. They reduced the ratio to

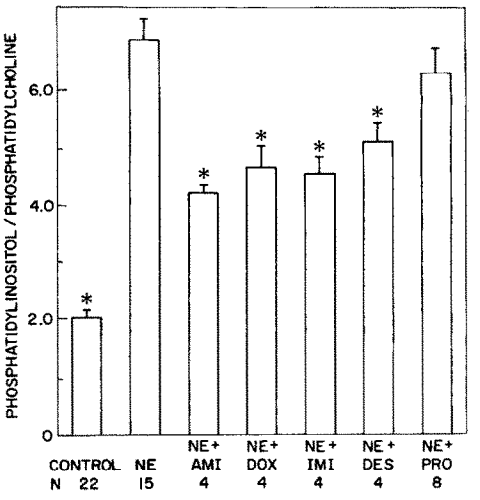

Fig. 1. Inhibition by tricyclic antidepressants of norepinephrine-stimulated phosphatidylinositol metabolism. Rat pineal glands were incubated with $^{32}P_i$ as described under Methods. (−)-Norepinephrine (NE), when present, was 10 μM. Amitriptyline (AMI), doxepin (DOX), imipramine (IMI), desipramine (DES) and protriptyline (PRO) concentrations were 1.5 μM. Phosphatidylinositol/phosphatidylcholine = ratio of P incorporation into the two phospholipids. Means ± S.E.M. are given. An asterisk (*) = $P < 0.05$, as compared to NE.

4.21 ± 0.14, 4.66 ± 0.36 and 4.53 ± 0.30 respectively.

While there are no marked differences in inhibitory potencies among individual drugs, the rank order of potency for alpha-adrenergic inhibition (tertiary > secondary amines) is in qualitative agreement with previous reports employing physiological responses [15] and radioligand binding [19]. However, the binding experiments with brain membrane fragments yield almost an order of magnitude difference in relative alpha-receptor affinities between amitriptyline and desipramine [19]. Differences in individual tricyclic potencies, as judged by inhibition of the phosphoinositide effect, are far less dramatic.

Lack of effect of cocaine in vitro

Many tricyclic antidepressants have long been known to inhibit the pre-synaptic reuptake of norepinephrine [20], which could thus, conceivably, influence quantitatively their apparent degree of inhibition of the norepinephrine-stimulated ^{32}P incorporation into phosphatidylinositol. To test whether the norepinephrine reuptake system of the pre-synaptic terminals which remain attached to the pineal gland after its removal from the brain could affect the net phosphoinositide effect induced by norepinephrine, 10 μM cocaine, a known pre-synaptic norepinephrine reuptake inhibitor, was added to the incubation medium. Since cocaine is a local anesthetic, it has the potential to alter phospholipid metabolism directly at high concentrations [21]. However, no effect on any of the five major phospholipids could be observed in the presence of cocaine (Table 1). Incorporation ratios elicited by 2 or 10 μM norepinephrine were not influenced significantly by the addition of 10 μM cocaine. From this observation it appears that pre-synaptic reuptake of norepinephrine, under the incubation conditions employed, does not

Table 1. Phosphoinositide effect in the presence of cocaine*

Norepinephrine (μM)	Cocaine (μM)	PhI/PhC+
0	0	2.26 ± 0.28
0	10	2.63 ± 0.12
2	0	1.98 ± 0.22
2	10	2.35 ± 0.21
10	0	6.50 ± 0.70
10	10	7.16 ± 0.76

*Rat pineal glands were incubated for 1 hr with $^{32}P_i$ (10 μCi), as described under Methods. Extracted lipids were separated by thin-layer chromatography and individual spots were counted. Means ± S.E.M. are given. In control incubations, incorporation (pmoles P/gland/hr) was: phosphatidylinositol, 71.4 ± 10.0; and phosphatidylcholine, 31.4 ± 1.0.

+ PhI/PhC = ratio of incorporation of ^{32}P into phosphatidylinositol to that into phosphatidylcholine.

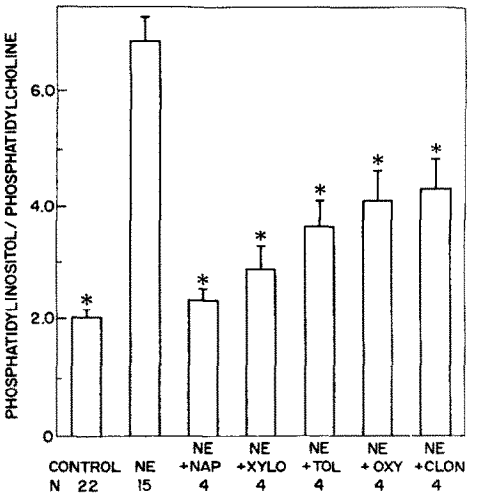

Fig. 2. Inhibition by imidazolines of norepinephrine-stimulated phosphatidylinositol metabolism. (−)-Norepinephrine (NE), when present, was 10 μM. Naphazoline (NAP), xylometazoline (XYLO), tolazoline (TOL), oxymetazoline (OXY) and clonidine (CLON) concentrations were 2.0 μM. Phosphatidylinositol/phosphatidylcholine = ratio of P incorporation into the two phospholipids. Means ± S.E.M. are given. An asterisk (*) = $P < 0.05$, as compared to NE.

influence the availability of added norepinephrine for the stimulation of ^{32}P incorporation into phosphatidylinositol *in vitro*. Thus, the magnitude of inhibition of the phosphoinositide effect by each of the tricyclics is the result of direct action on the post-synaptic alpha-receptor and is not a reflection of reuptake inhibition.

Inhibitory effects of imidazolines

All of the imidazolines tested at 2 μM antagonized the phosphoinositide effect elicited by 10 μM norepinephrine (Fig. 2). The rank order of potency of inhibition is: naphazoline > xylometazoline > tolazoline > oxymetazoline > clonidine, reducing the incorporation ratio to 2.34 ± 0.20, 2.90 ± 0.40, 3.63 ± 0.43, 4.11 ± 0.55 and 4.31 ± 0.55 respectively. Because imidazolines, in general, have agonist activities at peripheral alpha-adrenergic receptors [16], as well as at the pre-synaptic alpha-adrenergic receptor of the pineal gland [22], a few of the imidazolines were tested alone at 5 μM for intrinsic activity. Neither oxymetazoline, clonidine nor naphazoline exhibited intrinsic alpha-activity at this concentration. These data are similar to those of a recent report which shows imidazolines to be antagonistic at post-synaptic alpha-adrenergic receptors in the CNS [23]. These results support further our earlier conclusions that the phosphoinositide effect found in the pineal gland is probably post-synaptic in nature.

Competitive inhibition by amitriptyline and oxymetazoline

Amitriptyline and oxymetazoline, chosen as representatives of the tricyclics and imidazolines tested, were examined further to determine their mode of inhibition of the pineal alpha-receptor. Both compounds shifted the norepinephrine dose–response curve to the right in a parallel manner, suggesting a competitive type of inhibition for each (Fig. 3). The shifts were statistically significant at the 95 per cent confidence level calculated using the ordinate intercepts of the straight lines obtained by linear regression analysis.

DISCUSSION

The enhanced turnover rate of phosphatidylinositol in response to the action of a neurotransmitter (the phosphoinositide effect) appears to be tightly coupled to the degree of activation of post-synaptic alpha-

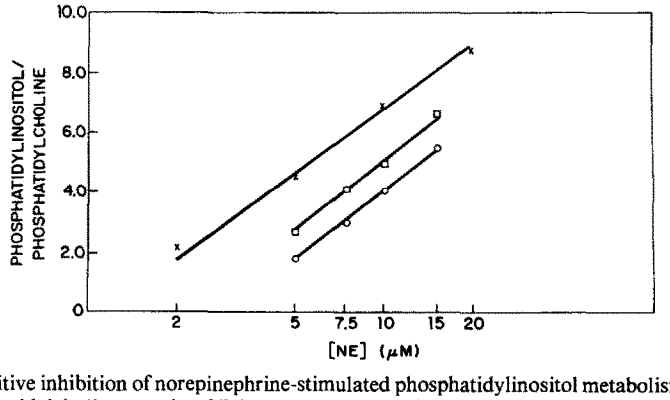

Fig. 3. Competitive inhibition of norepinephrine-stimulated phosphatidylinositol metabolism. Phosphatidylinositol/phosphatidylcholine = ratio of P incorporation into the two phospholipids. Key: (×) norepinephrine; (□) norepinephrine + 2 μM oxymetazoline; and (○) norepinephrine + 1 μM amitriptyline.

adrenergic receptors of the pineal gland [7]. Analogous effects have also been reported for a wide variety of other tissues [6]. A substantial amount of evidence suggests that the function of the alpha-receptor is to regulate transmembrane calcium flux [8]. There is only indirect evidence, however, which suggests that a change in phosphatidylinositol metabolism is a necessary intermediate biochemical event linking alpha-receptor activation to increased membrane permeability to calcium. Nevertheless, without direct evidence for the physiological consequences of the phosphoinositide effect, the selectively enhanced turnover of phosphatidylinositol can be utilized in itself as a biochemical response for pharmacologically evaluating drug activity at the post-synaptic alpha-adrenergic receptor. Thus, there exists the possibility of confirming or comparing results from such techniques as radioligand binding and physiological assays for alpha-activity with an additional biochemical response such as the phosphoinositide effect which presumably occurs as a consequence of transmitter-receptor recognition.

The post-synaptic alpha-adrenergic receptor of the pineal gland seems to exhibit properties similar to both the CNS and peripheral post-synaptic alpha-receptors. Thus, through the use of the phosphoinositide effect it is possible to show that the pineal alpha-receptor can be antagonized by tricyclic antidepressants, which is consonant with their inhibitory effects on the alpha-adrenergic receptors of rat cerebral cortical membrane fragments, as judged by the results of binding studies with [^{3}H]WB4101 [19], and the accumulation of cyclic AMP elicited by norepinephrine and mediated by alpha-adrenergic receptors in rat cerebral cortical slices [24]. Peripherally, tricyclic antidepressants antagonize norepinephrine-induced aortic muscle contractions which also involve alpha-adrenergic receptors [15]. In both peripheral and CNS tissues, tertiary amine tricyclics such as amitriptyline exhibit stronger alpha inhibitory potency than those tricyclics containing a secondary amine moiety such as desipramine and protriptyline. The pineal gland responds to tricyclic antidepressants in a similar manner (Fig. 1). Although tricyclic drugs are known to inhibit norepinephrine reuptake [20], the difference in inhibitory potencies of tertiary and secondary tricyclics is due to their direct post-synaptic action since the inhibition of pre-synaptic reuptake of norepinephrine does not seem to affect the net post-synaptic alpha-response to a given concentration of norepinephrine (Table 1). Analogous observations have also been reported for the post-synaptic beta-adrenergic receptor of the intact pineal gland [25]. The pre-synaptic reuptake system for norepinephrine may already be saturated *in vitro* upon exposure to exogenous norepinephrine, thereby masking any effect of reuptake inhibitors such as cocaine. Furthermore, destruction of pre-synaptic nerve terminals with 6-hydroxydopamine had no effect on the magnitude of the phosphoinositide effect in the pineal upon stimulation with norepinephrine [11]. Thus, the phosphoinositide effect *in vitro* does not appear to be modulated by a pre-synaptic component.

Alpha-receptors of various tissues seem to interact in a similar fashion with tricyclic antidepressants. This does not appear to be true, however, for the imidazolines which have been shown to be agonists in peripheral tissues [16, 26]. In the CNS their mode of action remains in dispute. Imidazolines are considered agonists at CNS alpha-adrenergic receptors which regulate blood pressure [26]. In addition, radioligand binding studies of cerebral cortical membrane fragments suggest an agonistic role for imidazolines [12]. Our results differ somewhat from these studies. At 5 μM concentrations, neither oxymetazoline, clonidine nor naphazoline exhibited intrinsic agonist activities, while they substantially antagonized the phosphoinositide effect elicited by norepinephrine (Fig. 2). When clonidine was tested in this system at higher concentrations, it acted as a partial α-agonist between 10 and 300 μM. Indeed, at 100 μM it reduced the stimulation achieved with 30 μM norepinephrine by 50 per cent (unpublished results). The action of imidazolines as described here is more consistent with their ability to effectively antagonize the norepinephrine-stimulated accumulation of cyclic AMP occurring through the activation of post-synaptic alpha-adrenergic receptors of cerebral cortical slices [23, 27]. Furthermore, clonidine and other imidazolines antagonize the post-synaptic alpha-adrenergic stimulation of K^+ release from dispersed rat parotid cells [28]. It would be of interest in future studies to investigate the influence of imidazolines on the phosphoinositide effect in smooth muscle preparations where most imidazolines display alpha-adrenergic agonist activities.

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REFERENCES

1. G. W. Pasternak and S. H. Snyder, *Molec. Pharmac.* **10**, 183 (1974).
2. A. Leon, D. Benvegnu, G. Toffano, P. Orlando and P. Massari, *J. Neurochem.* **30**, 23 (1978).
3. R. S. Aronstam, L. G. Abood and J. Baumgold, *Biochem. Pharmac.* **26**, 1689 (1977).
4. K. Von Hungen, S. Roberts and D. F. Hill, *J. Neurochem.* **22**, 811 (1974).
5. N. J. Swistocki and J. Tierney, *Biochemistry* **12**, 1862 (1973).
6. R. H. Michell, *Biochim. biophys. Acta* **415**, 81 (1975).
7. J. Eichberg, H. M. Shein, M. Schwartz and G. Hauser, *J. biol. Chem.* **248**, 3615 (1973).
8. L. M. Jones and R. H. Michell, *Biochem. Soc. Trans.* **6**, 673 (1978).
9. G. Hauser, H. M. Shein and J. Eichberg, *Nature, Lond.* **252**, 482 (1974).
10. G. Hauser, M. S. Nijjar, T. L. Smith and J. Eichberg, in *Cyclitols and Phosphoinositides* (Eds W. W. Wells and F. Eisenberg, Jr.), p. 167. Academic Press, New York (1978).
11. J. Eichberg, H. M. Shein and G. Hauser, *Biochem. Soc. Trans.* **1**, 352 (1973).
12. D. C. U'Prichard, D. A. Greenberg and S. H. Snyder, *Molec. Pharmac.* **13**, 454 (1976).
13. P. Skolnick, L. P. Stalvey, J. W. Daly, E. Hoyles and J. N. Davis, *Eur. J. Pharmac.* **47**, 201 (1978).
14. G. Kunos, *A. Rev. Pharmac. Toxic.* **18**, 291 (1978).
15. E. Elonen, M. J. Mattila and L. Saarnivaara, *Eur. J. Pharmac.* **28**, 178 (1974).
16. J. Sanders, D. D. Miller and P. N. Patil, *J. Pharmac. exp. Ther.* **195**, 362 (1975).
17. T. L. Smith and G. Hauser, *Soc. Neurosci. Abstr.* **4**, 522 (1978).
18. J. Folch, M. Lees and G. H. Sloane Stanley, *J. biol. Chem.* **226**, 497 (1957).

19. D. C. U'Prichard, D. A. Greenberg, P. P. Sheehan and S. H. Snyder, *Science* **199**, 197 (1978).
20. S. B. Ross and A. L. Renyi, *Eur. J. Pharmac.* **2**, 181 (1967).
21. J. Eichberg and G. Hauser, *Biochem. biophys. Res. Commun.* **60**, 1460 (1974).
22. F. Pelayo, M. L. Dubocovich and S. Z. Langer, *Eur. J. Pharmac.* **45**, 317 (1977).
23. P. Skolnick, J. W. Daly and D. S. Segal, *Eur. J. Pharmac.* **47**, 451 (1978).
24. G. C. Palmer, *Neuropharmacology* **15**, 1 (1976).
25. T. Deguchi and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **69**, 2208 (1972).
26. W. Hoefke, W. Kobinger and A. Walland, *Arzneimittel-Forsch.* **25**, 786 (1975).
27. P. Skolnick and J. W. Daly, *Eur. J. Pharmac.* **39**, 11 (1976).
28. J. N. Davis and W. Maury, *J. Pharmac. exp. Ther.* **207**, 425 (1978).