J. Pharm. Pharmacol. 1987, 39: 68–71
Communicated June 13, 1986

Evidence for spare α_1-adrenoceptors for the accumulation of inositol phosphates in smooth muscle

ANTHONY W. FOX*, PAUL A. FRIEDMAN†, *Clinical Pharmacology/Toxicology Unit, Departments of Medicine, Harvard Medical School and Beth Israel Hospital, Boston, MA 02215, USA*

The accumulation of inositol phosphates (IP) in smooth muscle from rat vas deferens and caudal artery was maximally increased 3- to 4-fold in response to exposure of the tissues to 100 μM noradrenaline. Clonidine (up to 3 mM) was a partial agonist. Pretreatment of the tissues with the irreversible α-adrenoceptor antagonist phenoxybenzamine (0·3–10 μM) shifted the noradrenaline concentration-response curve to the right before depressing the maximum. The maximum of the clonidine concentration-response curve was depressed without significant change in the EC50 by the same treatment. These data, which are most easily interpreted as demonstrating the presence of a receptor reserve for IP accumulation, are discussed.

Inositol phosphates (IP) are products of the catabolism of phosphatidylinositol (PI) phosphates (Hokin & Hokin 1953). Drug-receptor interactions enhance this catabolism (Michell 1975; Berridge 1984). Concentrations of IP in rat vas deferens and caudal artery increase in response to adrenoceptor agonist drugs (Canessa de Scarnati & Lapetina 1974; Fox et al 1985). In both these tissues, there are populations of α_1-adrenoceptors; there also appears to be a receptor reserve for contraction (Minneman et al 1983; Minneman & Abel 1984). The present study was designed to investigate whether such a reserve exists for the production of inositol phosphates in these tissues. Concentration-response curves (CRCs) to a full agonist, noradrenaline, and a partial agonist, clonidine, were constructed in a classical fashion with and without tissue pretreatment with the irreversible α-adrenoceptor antagonist phenoxybenzamine (Nickerson 1956).

Methods

Experiments were conducted largely as described by Fox et al (1985), with techniques adapted from those of Berridge et al (1982). Reserpinized rats (Sprague-Dawley, Charles River, Wilmington, MA) were killed by cervical dislocation, and the vasa deferentia and caudal arteries removed to Krebs Ringer bicarbonate buffer (KRB) which was kept equilibrated with an atmosphere of 95% O_2 and 5% CO_2 at 37 °C. The vasa were carefully stripped of adherent blood vessels and the connective tissue sheath and divided into 2 mm rings. The caudal arteries were cut into 15 mm lengths. 30 min was allowed for equilibration with the KRB(20 °C), then tissues were immersed in KRB at 37 °C for 20 min or in KRB containing phenoxybenzamine (0·3–10 μM). After thorough washing (4 washes in at least 50 mL KRB at 20 °C, 10 min for each wash) one ring of vas, or length of artery, was placed in 0·3 mL KRB containing 10 mM LiCl, 2 μM imipramine, the agonist under investigation, and 0·5 μCi of tritiated inositol (Amersham). Solutions of noradrenaline (1 mM) were prepared initially with 1 mg mL^{-1} ascorbic acid and diluted as required. Blank tubes lacking only tissue, and control tubes lacking agonists were assayed simultaneously with all experiments. Tissues were incubated for 2 h at 37 °C under 95% O_2, 5% CO_2; methanol in chloroform (2 mL, 2 : 1 v/v) was added and the tissues homogenized. More chloroform (0·63 mL) and water (1·26 mL) were added to form two layers. The aqueous phase was passed over an anion exchange column (9 mm, 0·375 mL of packed Dowex Ag 1-X8 resin, 100–200 mesh) in the formate form (Berridge et al 1982). Columns were washed with 40 mL unlabelled 5 mM myo-inositol, and were usually eluted with 2 mL 1 M ammonium formate in 0·1 M formic acid; some columns were sequentially eluted with increasing ionic strengths of borate and formate to partially separate IP (Brown et al 1984a; Fox et al 1985). Eluted IP were estimated by scintillation counting at about 40% efficiency. Mean values from blank tubes were deducted from all CRCs. Results were collected and analysed as counts min^{-1} without further transformation. The mixture of IP was usually used as a measure of response size. IP were not separated routinely in these experiments because with this technique the ratio of phosphates is constant amongst agonists and response sizes (Fox et al 1985). All values of EC50 are expressed as geometric mean ± s.e.

Results

Experiments in the presence of 10 μM noradrenaline showed IP accumulation to be linear with time up to 2·5 h.

* Present address and correspondence: Rm 227 Clinical Pharmacology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA.
† Present address: Merck, Sharp and Dohme Research Laboratories, WP-26, Room 285, West Point, PA 19486, USA.

IP accumulation was also linear with the amount of tissue in each assay tube, up to 4 rings of vas or 45 mm length of artery. In the absence of agonist only small amounts of IP accumulated. Exposure to noradrenaline produced responses 3–4 times control. Clonidine was found to be a partial agonist with maximal response 70 ± 6% of noradrenaline in the vas and 92 ± 8% in the artery (n = 6). Ratios of monophosphate(IP1) to bis- and tris-phosphates (IP2 + IP3) (mean ± s.e.m., n = 6) for noradrenaline were 56·7 ± 9·9 : 43·0 ± 3·5, and for clonidine 52·7 ± 4·1 : 47·4 ± 5·2.

Fig. 1 (rings of vas) shows CRCs to clonidine with and without pretreatment with three concentrations of phenoxybenzamine. Increasing concentrations of phenoxybenzamine reduced the maximal response, but there was no significant change in EC50 from the control value (log M, mean ± s.e.m., −6·23 ± 0·22). Similar data (not shown) were obtained from caudal artery (control EC50 (log M), −6·31 ± 0·12).

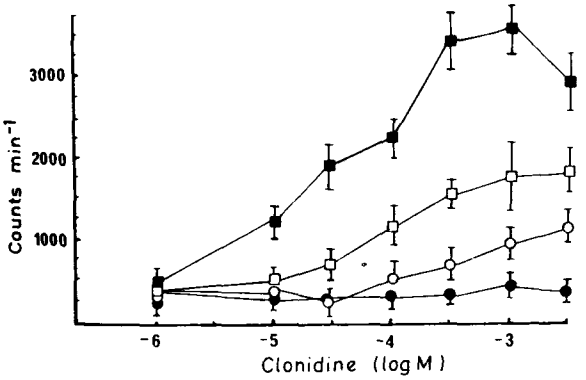

FIG. 1. Clonidine concentration-response curves (CRCs) for inositol phosphates accumulation in 2 mm rings of rat vas deferens. Experimental details are given in the text. Mean ± s.e.m. counts min^{-1} recovered are shown for tissues pre-incubated without (■, n = 19) and with phenoxybenzamine at the following concentrations: 0·3 μM (□, n = 8), 1·0 μM (○, n = 7), and 3·0 μM (●, n = 8). There are no significant differences between EC50 values for each CRC.

Fig. 2 (vas) and Fig. 3 (artery) show data from similar experiments using noradrenaline as the agonist. In both tissues the lowest concentration of phenoxybenzamine produced an increase in the EC50 without change in the maximal response size. After the higher concentrations of phenoxybenzamine, there were reduced maximal responses without significant change in EC50. Control log EC50 in vas was −5·06 ± 0·06 and in artery −5·61 ± 0·08 (a significant difference by *t*-test, $P < 0·05$). After treatment with phenoxybenzamine at concentrations sufficient to reduce the maximal response (demonstrating the absence of a receptor reserve), the EC50 in vas was −3·55 ± 0·05 and in artery −3·61 ± 0·06 (no significant differences between tissues or concentrations of phenoxybenzamine).

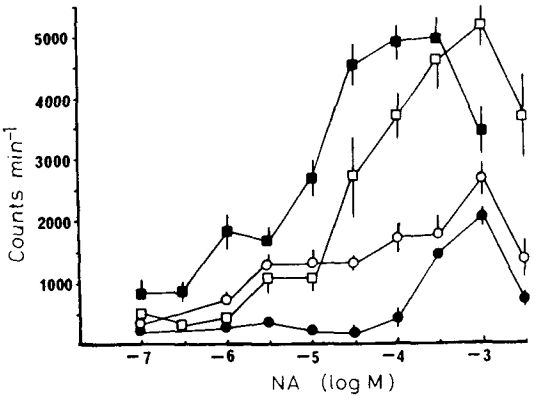

FIG. 2. Noradrenaline concentration-response curves for inositol phosphates accumulation in 2 mm rings of rat vas deferens, as described in the text. Mean ± s.e.m. counts min^{-1} recovered are shown for control tissues (■, n = 16) and those pretreated with phenoxybenzamine at the following concentrations: 1·0 μM (□, n = 8), 3·0 μM (○, n = 8), and 10 μM (●, n = 8). There are significant differences in EC50 between control and all phenoxybenzamine pretreatments (*t*-test, $P < 0·05$). There are no significant differences between EC50 values after phenoxybenzamine pretreatments.

All these experiments were conducted in the presence of 2 μM imipramine to block possible uptake of agonists during the incubation. However, control experiments produced no difference in noradrenaline EC50 with or without IMI in either tissue (data not shown). Another set of control experiments demonstrated no change in maximal response size or EC50 for a similar response to

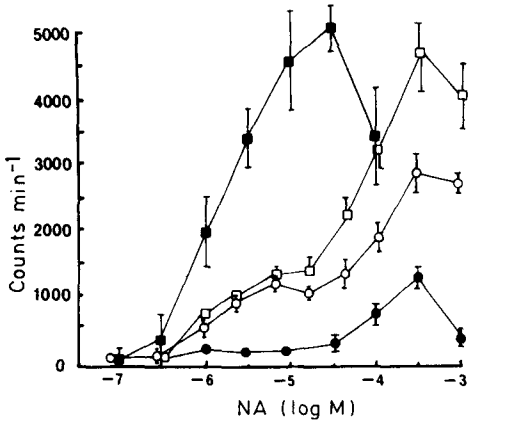

FIG. 3. Noradrenaline concentration-response curves for inositol phosphates accumulation in 15 mm lengths of rat caudal artery, as described in the text. Counts min^{-1} recovered (mean ± s.e.m.) related to log clonidine concentration for control tissues (■, n = 8), and after pretreatment with phenoxybenzamine at the following concentrations: 0·3 μM (□, n = 8), 1·0 μM (○, n = 8), and 3·0 μM (●, n = 8).

arg-vasopressin, with and without pretreatment with 3 μM phenoxybenzamine (maximal responses 2565 ± 230 counts min^{-1} control, 2920 ± 314 counts min^{-1} pretreated, log EC50: −8·92 ± 0·09 control, −9·01 ± 0·20 pretreated, n = 6 throughout).

Discussion

A partial agonist is unable to evoke a maximal pharmacological response; response size, according to theory, is then linearly related to the size of the receptor population activated. By definition, a receptor reserve can be proposed only for a full agonist that can evoke a maximal response by activating a fraction of the total receptor population. In the present study, all concentrations of phenoxybenzamine reduced the maximal responses to clonidine without changing the EC50. This is expected in theory for a partial agonist. However, with the full agonist noradrenaline, inactivation of a fraction of the adrenergic receptor population with phenoxybenzamine (1 μM in the vas and 0·3 μM in the caudal artery) did not prevent a maximal response, but resulted in an increase in EC50. Higher concentrations of phenoxybenzamine then reduced the maximal response to noradrenaline without change in the EC50. These data characterize the presence of an apparent receptor reserve for noradrenaline, for this response, in these tissues. Control noradrenaline EC50 values were greater in the vas than in the artery. This might be due to a greater receptor reserve in the artery.

There was also no effect of 3 μM phenoxybenzamine pretreatment on a similar response to arg-vasopressin in the caudal artery, indicating that there was no non-specific effect of phenoxybenzamine on IP production. This check was made because of the relatively high concentrations of phenoxybenzamine used in these experiments.

The EC50 values for both clonidine and noradrenaline after phenoxybenzamine treatment were consistent between tissues. This suggests that the EC50 is related to some constant. This is likely to be the affinity of agonist for receptor, which might be expected to be independent of both tissue and presence of phenoxybenzamine. However, the greatest EC50 values in these data are about 10 times the published K_d values from radioligand experiments and K_{act} measurements in contractile studies in the same tissues (Kenakin 1984; Abel & Minneman 1986). This systematic discrepancy could be due to a number of factors. The absence of an effect of imipramine on the control noradrenaline CRCs eliminated one possibility, that of competitive blockade by imipramine. Unpublished data (Fox & Minneman 1984) demonstrated no effect of 10 mM lithium on the binding of the radioligand [^{125}I]BE 2254 (2-[β-(4-hydroxyphenyl)ethylaminomethyl]tetralone) to α-adrenoceptors in these tissues. There may be a problem of access of agonist to receptor. For example, only the outer, longitudinal fibres of the vas contribute to a contraction, whereas a biochemical response such as IP accumulation may occur in the whole thickness of the tissue.

These data contrast with other reports in which no receptor reserve for IP production has been observed (Michell et al 1976; Berridge et al 1982; Fisher et al 1983; Amitai et al 1984; Minneman & Johnson 1984; Brown et al 1984b). Berridge et al (1982) showed a receptor reserve using 5-hydroxytryptamine for secretion, but not for IP accumulation, in the blowfly salivary gland.

Kenakin (1984) has discussed three mechanisms by which an apparent receptor reserve may be observed. These are (i) the response being produced by an enzyme cascade (e.g. Goldberg 1975), (ii) the tissue acting as a syncytium (e.g. Venter 1978), and (iii) saturation of the transduction mechanism by great density of activated receptors (Stephenson 1956; Minneman et al 1983). In the tissues used for the present study, to propose an enzyme cascade would be to propose a biochemical mechanism unlike any other reported, and with no evidence. Syncytial function would seem unlikely because if there is an access problem of agonist for receptor then this suggests that the agonist needs to have access to all parts of the tissue. Great receptor density (and substantial efficacy of noradrenaline) would seem the most likely alternative. Amitai et al (1984) reported no receptor reserve in cultured smooth muscle cells. However, the receptor density in that study (106 fmol (mg protein)$^{-1}$) was much lower than that for the intact organ (215 fmol mg^{-1}; Minneman et al 1983). Furthermore, receptor density in the homogenized vas or artery may be at a minimum because of the contribution to the protein concentration from connective tissues and non-contractile parts of the organ.

We thank Judith Hinchey for her excellent technical assistance, and Ms D. A. McDermott of Smith, Kline and French Laboratories for a gift of phenoxybenzamine. A. W. F. is supported by a fellowship from Ciba-Geigy.

REFERENCES

Abel, P. W., Minneman, K. P. (1986) J. Pharmacol. Exp. Ther. in press

Amitai, G., Brown, R. D., Taylor, P. (1984) J. Biol. Chem. 259: 12519–12527

Berridge, M. J. (1984) Biochem. J. 220: 345–360

Berridge, M. J., Downes, C. P., Hanley, H. R. (1982) Ibid. 206: 587–595

Brown, E., Kendall, D. A., Nahorski, S. R. (1984a) J. Neurochem. 42: 1379–1382

Brown, E., Kendall, D. A., Nahorski, S. R. (1984b) Br. J. Pharmacol. 81: 154P

Canessa de Scarnati, O., Lapetina, E. G. (1974) Biochem. Biophys. Acta 360: 298–305

Fisher, S. K., Klinger, P. D., Agranoff, P. W. (1983) J. Biol. Chem. 258: 7358–7368

Fox, A. W., Minneman, K. P., Abel, P. W. (1985) Eur. J. Pharmacol. 116: 145–153

Goldberg, N. D. (1975) in: G. Weissman, R. Claiborne (eds) Cell Membranes: Biochemistry, Cell Biology, and Pathology, H.P. Publishing, New York pp 185–202

Hokin, M. R., Hokin, M. E. (1953) J. Biol. Chem. 203: 967–977

Kenakin, T. P. (1984) Pharmacol. Rev. 36: 165–222

Michell, R. H. (1975) Biochem. Biophys. Acta 415: 81–147

Michell, R. H., Jafferji, S. S., Jones, L. M. (1976) FEBS Lett. 69: 1–5

Minneman, K. P., Abel, P. W. (1984) Mol. Pharmacol. 25: 56–63

Minneman, K. P., Johnson, R. D. (1984) J. Pharmacol. Exp. Ther. 230: 317–323

Minneman, K. P., Fox, A. W., Abel, P. W. (1983) Mol. Pharmacol. 23: 359–368

Nickerson, M. (1956) Nature (Lond.) 178: 697–698

Stephenson, R. P. (1956) Br. J. Pharmacol. Chemother. 11: 379–395

Venter, J. C. (1978) Mol. Pharmacol. 14: 562–574

J. Pharm. Pharmacol. 1987, 39: 71–72
Communicated June 12, 1986

Letter to the Editor

Inhibition of amphetamine-induced locomotor activity by *S*-(+)-apomorphine: comparison with the action of *R*-(−)-apomorphine

W. H Riffee*, R. E. Wilcox, *Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, Texas 78712, USA*

Saari et al in 1973, reported the synthesis of the *S*-(+) isomer of apomorphine [*S*-(+)-APO] as well as indicating that the isomer was inactive in producing postural asymmetries in mice in which the caudate had been unilaterally lesioned. However, our laboratories have shown that *S*-(+)-APO is an effective antagonist of *R*-(−)-apomorphine [*R*-(−)-APO]-induced stereotyped verticalization (Riffee et al 1982) with an ED50 of 7·7 mg kg^{-1} (observed during the inhibition of the action of 5 mg kg^{-1} *R*-(−)-APO). Thus, the potency of *S*-(+)-APO in blocking stereotypic activity is similar to that by which *R*-(−)-APO induces such behaviour. Recently we have used the amphetamine-stimulated locomotor model (Riffee & Wilcox 1985) to demonstrate that *R*-(−)-APO has activity presynaptically which results in the inhibition of the activity of the amphetamine. The present study was conducted to investigate the action of the *S*-(+)-APO isomer in comparison with the action of *R*-(−)-APO on amphetamine-stimulated locomotor activity.

The naive male albino CD-1 mice, 20–30 g, used had continual access to food and water but were food-deprived 24 h before testing. A 12 h light/dark cycle (lights on at 0700 h) was maintained and all testing was done between the hours of 0900 and 1700 h. Drugs used in the experiment were *R*-(−)-APO (MacFarland Smith, Edinburgh, Scotland), *S*-(+)-APO (Research Biochemicals, Wayland, Mass.) and amphetamine sulphate (Sigma, St Louis, MO). Drugs were prepared without preservatives immediately before use.

Locomotor activity was measured as described earlier (Riffee & Wilcox 1985) using Digiscan infrared activity monitors (Omnitech Electronics, Columbus, OH). All animals were pretreated with saline (0·9% NaCl) and given a 1 h habituation to the test chambers. The mice were then administered amphetamine (2·5 mg kg^{-1}) and returned to the test environment. Fifteen minutes later, half of the mice received *S*-(+)-APO or *R*-(−)-APO and the other half received saline. Locomotor activity was recorded for an additional 45 min. Data from the detectors represented actual distance travelled (in inches) per 5 min period. A microprocessor, programmed by the manufacturer (Omnitech), integrates the various angles in which the animal moves so that actual distance travelled can be determined. Sequential infrared beams must be interrupted for distance travelled to be registered. Continuous interruption of one beam by a behaviour such as head-bobbing would not be recorded as horizontal movement. Data analysis was done using analysis of variance with appropriate post hoc tests for significance (Wilcox et al 1979).

* Correspondence.