Biochemical Pharmacology, Vol. 33, No. 7, pp. 1143–1145, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

SHORT COMMUNICATIONS

Desensitization of the alpha$_1$ adrenoceptor system in vascular smooth muscle

(*Received* 7 *July* 1983; *accepted* 26 *September* 1983)

At present, little is known about the influence of prolonged activation of post-synaptic alpha$_1$ adrenoceptors on post-receptor mechanisms and vascular reactivity. Increased receptor activation may occur via increases in sympathetic activity or plasma noradrenaline concentration, as have been suggested to occur in some patients with essential hypertension [1], experimental hypertension [2] or in phaeochromocytoma [3]. Direct pharmacological assessment of post-synaptic alpha$_1$ adrenoceptor sensitivity in blood vessels is complicated by structural alterations in hypertrophied vessels due to hypertension which results in non-specific increases in sensitivity to all agents which induce vessel contracture [4]. It has been suggested that alpha adrenoceptor responsiveness of vessels in patients with phaeochromocytoma may be reduced due to high circulatory levels of catecholamines [3]. This hypothesis is supported by recent experiments on rabbit aortic strips which exhibited a reduced sensitivity to alpha$_1$ adrenoceptor mediated contracture upon repeated exposure to noradrenaline [5]. More recently, Colucci *et al.* [6] have demonstrated changes in alpha$_1$ adrenoceptor affinity as well as in receptor numbers under conditions which alter environmental catecholamine numbers. The aim of our experiments was to examine in more detail the effect of prolonged exposure of vascular smooth muscle cells to noradrenaline on alpha$_1$ adrenoceptor properties. Experiments were carried out in cultured cells in order to exclude any influence of secondary *in vivo* effects ('reflex effects') on vascular alpha$_1$ adrenoceptors which may not be directly associated with the interaction between noradrenaline and vascular alpha$_1$ adrenoceptors.

Materials and methods

Materials. Biochemicals were purchased from the Sigma Chemical Co. (St. Louis, MO) and media and reagents for cell culture were from CSL Laboratories (Parkville, Australia). [^{125}I]-2-[β-(4-Hydroxyphenyl)-ethylaminomethyl]tetralone ([^{125}I]BE 2254) was purchased from New England Nuclear (Boston, MA) and all other radiochemicals were from Amersham International (Bucks., U.K.).

Growth of cells. The thoracic and abdominal aortic media of six 7-week-old rabbits were dispersed into single cells by collagenase and elastase and seeded into 10 plates at a concentration of 1×10^6 cells/10 ml of nutrient media which consisted of Medium 199 containing 10% foetal calf serum and 2 mM glutamine. The cells were used after 6–7 days in culture. Histochemical staining of the monolayer of confluent cells with FITC-labelled antibodies specific for smooth muscle myosin indicated greater than 99% of the smooth muscle cells were contractile at this time [7]. The yield of cell protein resulting from one culture averaged *ca.* 10 mg protein as determined by the method of Lowry *et al.* [8].

'In vitro' *desensitization.* After 7 days in culture, the growth medium was replaced with Medium 199 containing only 5% foetal calf serum. A freshly prepared solution of noradrenaline dissolved in the culture medium was added to 2–5 plates so that its final concentration was 10^{-4} M. Plates were re-incubated for 24 hr prior to determination of alpha$_1$ adrenoceptor properties and responses.

[^{125}*I*]*BE 2254 specific binding.* Control and noradrenaline exposed cells were washed free of culture medium with 3×10 ml Dulbecco's phosphate buffer [9], scraped from the plates and transferred into 20 ml of ice-cold Dulbecco's phosphate buffer. The cells were then further washed to remove the last traces of noradrenaline by resuspension in Dulbecco's phosphate (2×20 ml) and centrifugation at 1000 *g* for 15 min. The final pellets were resuspended in 2 ml 50 mM sodium phosphate buffer (pH 7.5) and homogenized with a Polytron PT10 homogenizer (speed 5, 10 sec). The resultant homogenate was diluted to 8 ml and centrifuged at 10^5 *g* for 30 min. The pellets were resuspended in 8 ml of the homogenizing buffer and recentrifuged. The final pellets were resuspended at an average protein concentration of 3 mg/ml.

[^{125}I]BE 2254 specific binding was measured by incubating (25°) approximately 0.3 mg of particulate protein with [^{125}I] BE 2254, up to 500 pM in the absence and presence of 3.3×10^{-4} M noradrenaline as described previously [10]. In displacement studies the concentration of noradrenaline ranged from 10^{-7} to 10^{-3} M and the [^{125}I]BE 2254 concentration averaged 39 ± 2 pM. Bound [^{125}I]BE 2254 was separated from its free form by filtration under vacuum through Whatman GF/C glass filters which were washed twice with 10 ml of ice-cold 50 mM sodium phosphate (pH 7.5) incubating buffer. Saturation specific binding isotherms were analysed directly by computer as described by Parker and Waud [11], as well as using linearized transformations of the binding isotherms (Eadie–Hofstee plot) [12]. All results are means ± S.E. Statistical significance of differences between means was assessed by Student's paired *t*-test [13].

[^{32}P]*Phosphatidylinositol turnover.* Confluent plates of smooth muscle cells were rinsed three times with Krebs–Ringer–Tris (KRT) buffer of the following composition (mM): NaCl 120, $CaCl_2$ 1.4, KCl 5.2, $MgSO_4$ 1.4, Tris 5, pH 7.4. Washed cultures were pre-incubated for 1 hr at 37° in 4 ml KRT buffer containing 20 μCi ^{32}P as orthophosphate, washed free of unicorporated radioactivity and incubated at 37° for 40 min with 30 μM (±)-propranolol in the presence and absence of 10 μM (−)-adrenaline. Cells were harvested in 2×1 ml KRT buffer (4°) and isolated by centrifugation. Phospholipids were isolated by the method of Folch *et al.* [14] by partitioning between chloroform–methanol (2:1 by vol.) and 125 mM aqueous NaCl after homogenization (Polytron PT10, speed 5, 10 sec). Phosphatidylinositol was isolated by thin-layer chromatography on 0.25 mm silica gel plates (E. Merck, Darmstadt F.R.G.) sprayed with 5% aqueous magnesium acetate, dried for 1 hr at 120° and developed in chloroform–methanol–concentrated ammonia–water (65:35:3:2 by vol.). Spots were visualized after 16 hr autoradiography. Approximately 70% of the total extracted radioactivity was associated with a single spot which co-chromatographed with [^{3}H]phosphatidylinositol. Phosphatidylinositol was extracted from silica with methanol and after evaporation to dryness total phosphorus was assayed according to the method of Bartlett [15]. ^{32}P was determined by liquid scintillation counting of the entire product of the phosphorous assay using Instagel (Packard Inc., Downers Grove, IL).

Results and discussion

[^{125}I]BE 2254 bound to particulate protein of smooth muscle cells in a manner consistent with binding to alpha adrenoceptors. Since [^{125}I]BE 2254 has been reported to be an alpha$_1$ adrenoceptor radioligand [16, 17], we have assumed that its specific binding respresent binding to vascular alpha$_1$ adrenoceptors. Binding affinity of [^{125}I]BE 2254 averaged 55 ± 10 pM, which is in agreement with its binding affinity for alpha$_1$ adrenoceptors in other tissues [16]. In these calculations, no correction has been made for possible differences in the affinities of [^{125}I]BE 2254 enantiomers which are in dynamic equilibrium in solution [17]. Exposure of smooth muscle cells to 10^{-4} M noradrenaline for 24 hr reduced the number of [^{125}I]BE 2254 specific binding sites by 41% ($P < 0.01$) without significantly affecting the affinity (K_D) of [^{125}I]BE 2254 for this binding site. K_D averaged 56 ± 17 pM for control cells and 53 ± 8 pM for noradrenaline-exposed cells (P for difference > 0.05). Similarly, there was no difference in the ability of noradrenaline to displace specifically bound [^{125}I]BE 2254 from particulate fractions of control and noradrenaline-exposed cells (see Fig. 1, bottom panel). The K_i for noradrenaline averaged 1.75 ± 0.51 μM for control cells and 1.42 ± 0.15 μM for noradrenaline-exposed cells ($P > 0.05$).

The reduction in alpha$_1$ adrenoceptor numbers following exposure to noradrenaline was accompanied by a reduction in the extent of alpha$_1$ adrenoceptor mediated stimulation of [^{32}P]phosphatidylinositol turnover (Fig. 2, bottom panel). The degree of stimulation of phosphatidylinositol specific activity was reduced by an average of 67%; this was greater than the 41% decrease in alpha$_1$ adrenoceptor concentration measured under the same conditions. Basal rates of [^{32}P]phosphate incorporation into phosphatidylinositol were not affected, suggesting that the reduction in specific activity was not due to any non-specific inability of the cell to incorporate [^{32}P]phosphate into this phospholipid.

Colucci *et al.* [6] reported that prolonged adrenaline infusion in rats only reduced mesenteric artery alpha$_1$ adrenoceptor concentration whilst treatment with 6-hydroxydopamine or reserpine to deplete tissue catecholamines did not affect receptor number, but increased the affinity of alpha$_1$ adrenoceptors for adrenaline. On the basis of these results, they suggested that alpha$_1$ adrenoceptors of rat mesenteric artery under normal circumstances exist at maximum concentration and at lowest affinity for adrenaline, i.e. are 'up-regulated' with respect to number and 'down-regulated' with respect to affinity. In our smooth muscle cultures, there is no sympathetic innervation and hence alpha$_1$ adrenoceptors would be expected to be at a maximum concentration and highest affinity. One would therefore expect both a reduction in alpha$_1$ receptor number as well as affinity upon prolonged exposure to noradrenaline. Our results indicate that only receptor number is affected by prolonged exposure of smooth muscle cells to noradrenaline. Presumably, 6-hydroxydopamine or reserpine treatments are influencing alpha$_1$ adrenoceptor affinity indirectly in a manner unrelated to depletion of noradrenaline in sympathetic nerves associated with mesenteric arteries.

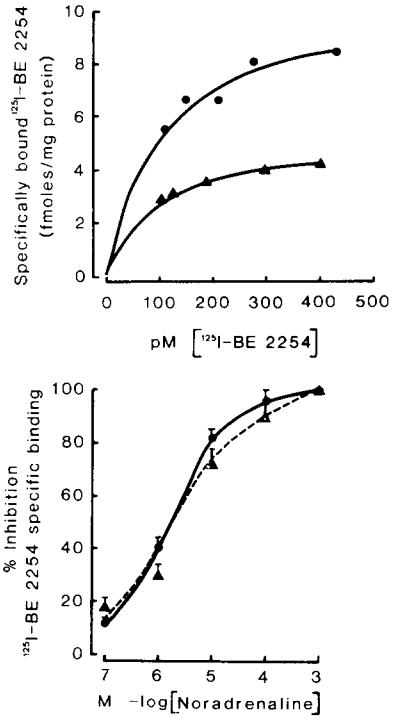

Fig. 1. Top panel: [^{125}I]BE 2254 specific binding to particulate fraction of control (●) and noradrenaline- (100 μM) exposed (▲) smooth muscle cells. Results are the means of duplicate determinations from a typical experiment. For control cells, B_{max} = 10.3 fmole/mg protein and K_D = 94 pM; for noradrenaline-exposed cells, B_{max} = 4.9 fmole/mg protein and K_D = 73 pM. Bottom panel: Inhibition of [^{125}I]BE 2254 specific binding by noradrenaline to particulate fraction of control (●) and noradrenaline-(100 μM) exposed (▲) smooth muscle cells. Results are the mean ± S.E. of experiments from four separate cultures.

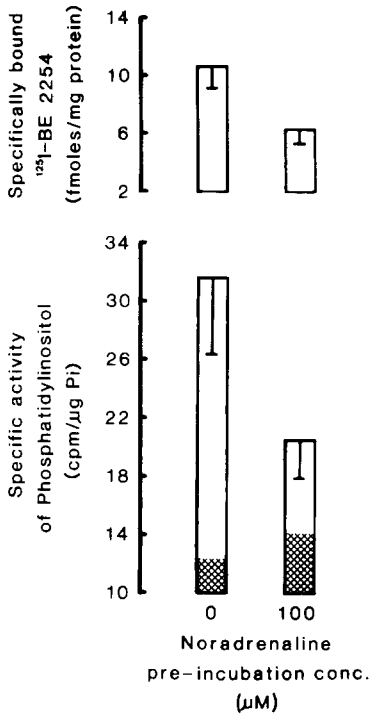

Fig. 2. Top panel: [^{125}I]BE 2254 specifically bound to particulate fraction of control and noradrenaline-exposed cells. Bottom panel: Specific activity of phosphatidylinositol in control or noradrenaline- (100 μM, 24 hr) exposed cells following stimulation for 40 min with (−)-adrenaline (10 μM) in the presence of (±)-propranolol (30 μM). Cells were labelled by incubation in [^{32}P]phosphate for 60 min. Hatched areas show specific activity of phosphatidylinositol in cells treated as above but not stimulated with adrenaline. Results are means ± S.E. of experiments from four separate cultures.

The reduction in alpha$_1$ adrenoceptor mediated [^{32}P]phosphatidylinositol turnover following prolonged exposure to noradrenaline is consistent with the observed reduction in receptor concentration. Increases in [^{32}P]phosphatidylinositol turnover due to alpha$_1$ adrenoceptor activation have been postulated to be secondary, in response to an increased rate of phosphatidylinositol hydrolysis by phospholipase C [18]. Harrington and Eichberg [19] have recently demonstrated the breakdown of phosphatidylinositol in a reconstituted cell-free preparation by an apparently alpha$_1$ adrenoceptor mediated mechanism. Whether the reduction in [^{32}P]phosphatidylinositol turnover demonstrated by us represents an inability of alpha$_1$ adrenoceptors to activate phospholipase C either directly or indirectly remains to be determined.

In summary, our experiments indicate that the concentration of alpha$_1$ adrenoceptors on vascular smooth muscle cells in culture is reduced following prolonged exposure to noradrenaline. There appears to be no significant change in the affinity of the remaining receptors for noradrenaline. This reduction in receptor number is accompanied by a reduction in alpha$_1$ adrenoceptor mediated turnover of cell [^{32}P]phosphatidylinositol. The greater magnitude of the reduction in [^{32}P]phosphatidylinositol turnover over the reduction in receptor number suggests that for this cell response there may be (i) spare alpha$_1$ adrenoceptors, or (ii) the process of alpha$_1$ adrenoceptor desensitization involves receptor uncoupling, possibly from phospholipase C as well as receptor loss.

Acknowledgement—This work was supported by a grant-in-aid from the National Heart Foundation of Australia.

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Biochemical Pharmacology, Vol. 33, No. 7, pp. 1145–1147, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

Noradrenergic inhibition of the nicotinically-stimulated release of acetylcholine from guinea-pig ileal synaptosomes

(*Received* 2 *June* 1983; *accepted* 8 *September* 1983)

Paton and co-workers [1, 2] first demonstrated that electrical stimulation of segments of the guinea-pig ileum produces contractions which are mediated by the release of acetylcholine (ACh) from nerve terminals of the myenteric plexus. Norepinephrine (NE) inhibits the contractions and the release of ACh by a mechanism which involves α-adrenergic receptors [3, 4]. Following Langer's proposal [5] that α-adrenoceptors are divided into the postsynaptic α_1-adrenoceptor and the presynaptic α_2-adrenoceptor, it was shown that the cholinergic activity in the intact ileal myenteric plexus is modulated by α_2-adrenoceptors [6–8].

We have described previously a preparation of synaptosomes derived from the myenteric plexus of the guinea-pig ileum [9]. Both 50 mM KCl and 10 μM 1,1-dimethyl-4-phenyl piperazinium (DMPP) increase the calcium-dependent release of [^{3}H]ACh from these synaptosomes [10], but only the DMPP-induced release is modulated significantly by oxotremorine [10] or adenosine [11]. Based on the ability of NE to inhibit both the electrically-stimulated and nicotinically-induced [12] release of ACh from the intact myenteric plexus, we decided to determine whether NE might modulate the nicotinically-stimulated release of [^{3}H]ACh from the synaptosomal preparation and, if so, to determine what type of receptor was involved.

Methods

Preparation of the P_2 fraction. The P_2 fraction was obtained from the guinea-pig ileum longitudinal muscle-myenteric plexus preparation as previously described [11].

Synthesis and release of [^{3}H]acetylcholine. The P_2 pellet was suspended in 5 ml of Krebs–Ringer bicarbonate buffer of the following composition (mM concentrations): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 25 and dextrose, 10. The buffer was gassed with 95% O_2–5% CO_2 to maintain a pH of 7.4. The tissue was