J. Pharm. Pharmacol. 1983, 35: 584–589
Received February 2, 1983

α-Adrenoceptor subtypes and Ca^{2+} mobilization the rabbit ear artery†

STEFANO MANZINI*, CARLO ALBERTO MAGGI AND ALBERTO MELI

Pharmacology Department, Research Laboratories, A. Menarini Pharmaceuticals, Via Sette Santi 3, 50131 Florence, Italy

The mobilization of cellular and extracellular Ca^{2+} pools by selective α_1-adrenoceptor (phenylephrine) and α_2-adrenoceptor (xylazine) agonists as well as noradrenaline was evaluated in rabbit ear artery. Noradrenaline and phenylephrine possess full instrinsic activity for both types of Ca^{2+} mobilization whereas xylazine up to 1 mM had only a limited contractile effect, being more effective in inducing extracellular Ca^{2+}-dependent response. However, extracellular Ca^{2+} was mobilized by xylazine in a concentration 20 times higher than that required to stimulate pre-junctional α_2-adrenoceptors. Noradrenaline (5 μM) and xylazine (1 mM) induced cellular and extracellular Ca^{2+}-dependent contractions which were prazosin-sensitive and yohimbine-resistant. Xylazine-induced contractile activity, particularly that dependent upon the extracellular Ca^{2+} pool, was markedly reduced by selective adrenergic denervation with 6-hydroxydopamine, but the actions of noradrenaline were unaffected. These results suggest that: (1) rabbit ear artery contain post-junctional α_1-adrenoceptor but not α_2-adrenoceptors; (2) stimulation of these α_1-adrenoceptors can account for the overall contractile activity of exogenously added noradrenaline and (3) stimulation of α_1-adrenoceptors results in mobilization of cellular as well as extracellular Ca^{2+} pools.

In some vascular smooth muscle, noradrenaline induces typical biphasic contractions dependent upon mobilization of both cellular and extracellular Ca^{2+}: initiation of tension is produced by the release of limited amounts of intracellular Ca^{2+} but maintenance of tension is primarily dependent upon influx of Ca^{2+} from the extracellular space (Bevan et al 1973; Deth & Van Breemen 1974; Deth & Casteels 1977; Karaki et al 1979).

On the basis of in-vivo (Drew & Whiting 1979: Docherty et al 1979; Timmermans et al 1979) and radioligand binding (U'Prichard & Snyder 1979; Hoffman et al 1979) studies, evidence has been presented indicating that vascular smooth muscle of several species contains a mixed population of postjunctionally located α_1- and α_2-adrenoceptors; activation of either subtype results in a vasoconstrictor response.

Although pressor responses due to activation of α_2-adrenoceptors can be easily obtained in in-vivo preparations, the presence of these receptors is difficult to demonstrate in isolated vascular preparations. Consequently it is still debatable whether or not there are differences in stimulus-contraction coupling and Ca^{2+} requirements for vasoconstriction between responses elicited by α_1- and α_2-adrenoceptors agonists (McGrath 1982). Recently it has been hypothesized that α_1-adrenoceptor activation may open potential independent calcium channels as well as determine the liberation of calcium from intracellular stores, whereas activation of α_2-adrenoceptors may cause only the opening of potential dependent channels for transmembrane Ca^{2+} influx (Langer & Shepperson 1982).

A newly developed in-vitro procedure (Manzini et al 1982a) makes it possible to obtain a separate quantitative analysis of the cellular and extracellular Ca^{2+}-dependent phases of contraction induced by noradrenaline. It appeared worthwhile to use this procedure to determine: (a) the α-adrenoceptor subtype through which noradrenaline exerts its effect; (b) whether or not stimulation of α_1-adrenoceptors mobilizes cellular and extracellular Ca^{2+} stores; and (c) whether activation of α_2-adrenoceptors is involved in the vasoconstriction response and, if they are, which types of Ca^{2+} pools are involved. Therefore, we set out to determine the effects of selective α_1- and α_2-adrenoceptor agonists (phenylephrine and xylazine, respectively) in mobil-

† Preliminary data were presented at the International Symposium on Calcium Modulators, Venice, June 17–18, 1982.

* Correspondence.

izing cellular and extracellular Ca^{2+} pools, and the effects of selective α_1- and α_2-adrenoceptor antagonists (prazosin and yohimbine, respectively) on noradrenaline-induced mobilization of cellular and extracellular Ca^{2+} pools.

METHODS

New Zealand male albino rabbits 2·5–3 kg, were anaesthetized with urethane (1·5 g kg^{-1}, i.p.) and heparinized (1000 i.u., i.v.). A 3 cm segment of central ear artery was dissected free from adhering tissues, cannulated at both ends with polyethylene cannulae and transferred to a 7 ml organ bath (at 37 °C); the volume of fluid in the bath was maintained constant by means of an overflow. The arterial segment was perfused intraluminally by means of a De Saga 131900 six-channel peristaltic pump at a rate of 5 ml min^{-1}, and extraluminal perfusion at a rate of 8 ml min^{-1} was by means of a Mariotte bottle. Both intraluminal and extraluminal perfusion fluids were gassed with 95% O_2 and 5% CO_2 and heated to 37 °C. Changes in intraluminal perfusion pressure, recorded by means of a pressure transducer attached to the inlet side of the perfusion system, were taken as an indirect measure of increase in arterial contraction above the resting tone. Resting perfusion pressure was found to be 29·4 ± 2·8 mm Hg (mean ± s.e., n = 33). After a 1–2 h stabilization period, the extraluminal and intraluminal normal Krebs solution was replaced by a high-K^+, Ca^{2+}-free solution which produced a rapid contraction with gradual return to resting values. This procedure is expected to deplete extracellular Ca^{2+}, to mobilize and subsequently to deplete Ca^{2+} bound to low affinity sites of the plasma membrane (Karaki & Weiss 1980), and to prevent any neuronal or electrical interference with drug-induced contractions. Five minutes later the intraluminal perfusion fluid was substituted for 3 min with the high-K^+, Ca^{2+}-free solution containing noradrenaline, phenylephrine, or xylazine at the desired concentration, which produced a contraction followed by return to resting values. Subsequent intra- and extraluminal perfusion with normal Krebs resulted in a further rapid contraction, which depends upon the presence of extracellular Ca^{2+} and is related to the concentration of the agonist previously added to the inner perfusion fluid. Agonist-induced contractions in high-K^+, Ca^{2+}-free medium have been assumed to be purely dependent on the cellular Ca^{2+} store since: (a) they are obtained in the absence of extracellular Ca^{2+} and in the presence of EDTA; (b) they can be elicited by drugs such as aminophylline, which are capable of releasing intracellular Ca^{2+} (Manzini et al 1982b) and (c) they can be antagonized by drugs such as procaine (Manzini et al 1982a) which prevent intracellular Ca^{2+} mobilization (Robinson & Sastry 1976; Hunter et al 1982).

The contraction which follows reperfusion with normal Krebs solution can be taken as an indirect functional measure of the agonist induced enhancement of Ca^{2+} conductance across the sarcolemma of smooth muscle cells and thus as an extracellular Ca^{2+}-dependent contraction since: (1) it becomes evident only when the preparation has been pre-exposed to drugs capable of opening receptor operated channels for transmembrane Ca^{2+} influx; (2) its height is strictly proportional to the Ca^{2+} concentration in the reperfusing medium (Manzini et al 1982a), and (3) it is selectively inhibited by Ca^{2+} entry blocking drugs such as verapamil (Manzini et al 1982a) or nifedipine (unpublished observation).

The peak amplitude and duration of each contraction were measured. Preliminary experiments showed that after a 25 min period of perfusion with normal Krebs solution, the agonists produced reproducible responses that were suitable for determination of non-cumulative concentration-response curves.

The effects of xylazine and phenylephrine were evaluated by taking as 100% the values relative to cellular and extracellular Ca^{2+}-dependent phases of responses to a supramaximal concentration of noradrenaline (5 μM) obtained in the same arterial segment. Modification of the response to noradrenaline (5 μM) and xylazine (1 mM) by prazosin and yohimbine was studied by the addition of the antagonist at the stated concentration in the inner perfusion fluid for 3 min before and during 3 min of challenge with noradrenaline and xylazine. The effects of antagonists on contractions induced by noradrenaline and xylazine were assessed on paired preparations from the same animal, one of which served as control. For evaluating the prejunctional activity of xylazine, preparations were excited by passage of current across platinum wire electrodes placed on both sides of the vascular segment. Rectangular pulses of 10 ms duration and supramaximal voltage were applied at 20 Hz for 3 s at intervals of 90 s. The indirect nature of the transmural field stimulation was previously confirmed by its abolition by phentolamine (1 μM; n = 4) and tetrodotoxin (6 μg ml^{-1}; n = 4).

In another set of experiments, central arteries of both ears of the same rabbit were excised, then, after

a 1 h stabilization period, one of them was perfused intraluminally for 30 min with Krebs solution containing 6-hydroxydopamine 400 mg litre^{-1} which, according to the method of Aprigliano & Hermsmeyer (1976), provided selective adrenergic denervation, while the other served as control. Two hours later, non-cumulative concentration–response curves for noradrenaline or xylazine were determined in parallel experiments. The efficiency of the denervation procedure was proved by preliminary experiments which showed a reduction of 93·6 ± 6·1% in the response to tyramine 10 μg ml^{-1} administered before and after treatment with 6-hydroxydopamine.

The standard Krebs solution had the following mM composition: NaCl 119, KCl 4·7, $MgSO_4$ 1·5, KH_2PO_4 1·2, $CaCl_2$ 2·5, $NaHCO_3$ 25, glucose 11: the high K^+, Ca^{2+}-free solution had the following mM composition: NaCl 69, KCl 54·7, $MgSO_4$ 1·5, KH_2PO_4 1·2, $NaHCO_3$ 25, glucose 11, EDTA 0·77.

Results are expressed as difference between intraluminal and relative basal pressure. All values are expressed as mean ± s.e. Statistical analysis of the data was performed by Student's *t*-test for unpaired data and, to evaluate the prejunctional activity of xylazine ED50 and 95% confidence limits were calculated according to Litchfield & Wilcoxon (1949).

Drugs employed were: (−)-noradrenaline base (Gianni), (±)-phenylephrine HCl (Serva), xylazine HCl (Rompun, Bayer), prazosin HCl (Fermion OY, Orion), yohimbine HCl (Aldrich), 6-hydroxydopamine HBr (Sigma), tyramine HCl (Sigma), phentolamine mesylate (Regitin, Ciba) and tetrodotoxin (Sankyo).

RESULTS

Replacement of normal Krebs with a high K^+, Ca^{2+}-free solution produced a transient rise in perfusion pressure (136·2 ± 8·4 mm Hg; n = 163) which returned to resting value within 2–3 min. Reperfusion with normal Krebs (8 min later) did not affect perfusion pressure unless intraluminal α-adrenoceptor agonists had been previously added to the medium. In earlier experiments it was demonstrated that intraluminal noradrenaline in high-K^+, Ca^{2+}-free solution produced a dose-related cellular Ca^{2+} dependent phasic contraction (Manzini et al 1982a, b); reperfusion with normal Krebs of noradrenaline-pretreated arterial segments resulted in a rapid extracellular Ca^{2+}-dependent contraction, the amplitude and duration of which were concentration related (Manzini et al 1982a).

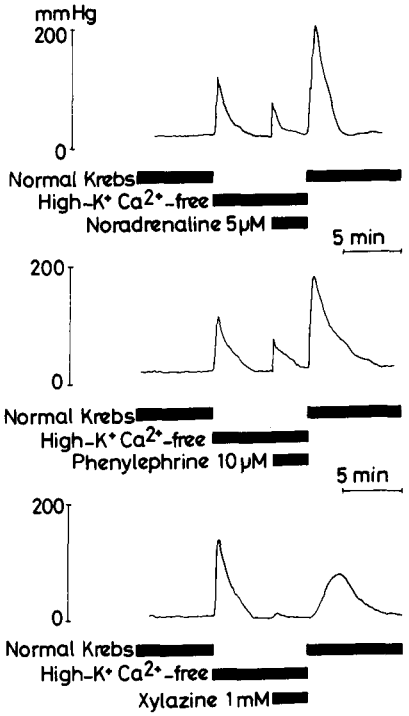

FIG. 1. Typical tracings of experiments illustrating the effect of intraluminal and extraluminal substitution of normal Krebs with high-K^+, Ca^{2+}-free medium, the subsequent intraluminal administration of noradrenaline (5 μM), phenylephrine (10 μM) or xylazine (1 mM), and then intraluminal and extraluminal reperfusion with normal Krebs.

Contractile activity of phenylephrine, xylazine and noradrenaline in high-K^+, Ca^{2+}-free solution and after reperfusion with normal Krebs

Fig. 1 shows typical tracings of contractions induced by noradrenaline, phenylephrine and xylazine in high-K^+, Ca^{2+}-free and after reperfusion with normal Krebs. For the sake of convenience, the contractions elicited in high-K^+, Ca^{2+}-free medium will be termed 'cellular Ca^{2+}-dependent' and those after reperfusion with normal Krebs solution as 'extracellular Ca^{2+}-dependent'. Unlike phenylephrine and noradrenaline, xylazine induced a barely detectable cellular Ca^{2+} dependent contraction and a slow onset long-lasting extracellular Ca^{2+} dependent contraction.

Fig. 2 shows the concentration-response curves for cellular and extracellular Ca^{2+} dependent contractions induced by phenylephrine and xylazine. Unlike phenylephrine, which possesses full intrinsic activity in triggering both types of Ca^{2+}-dependent contraction, xylazine showed weak potency and, with concentrations up to 1 mM, maximal responses were not obtainable.

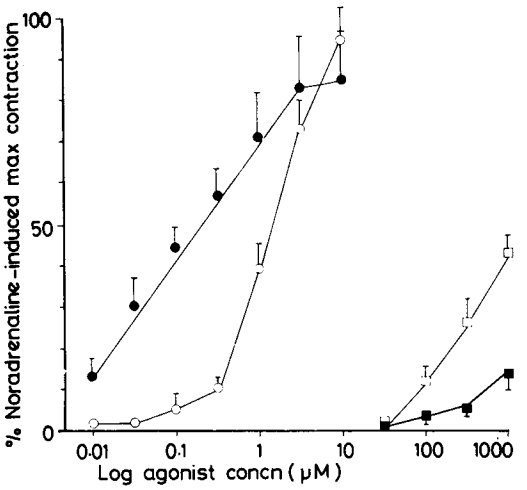

FIG. 2. Concentration–response curves for the amplitude of cellular (solid symbols) and extracellular (open symbols) Ca^{2+}-dependent contractions induced by phenylephrine (circles) and by xylazine (squares). The percentage of activity was calculated by taking as 100% the value of cellular and extracellular Ca^{2+}-dependent contractions induced by a supramaximal concentration of noradrenaline (5 μM) in the same arterial segments (see methods). Each point is the mean ± s.e. of at least six experiments.

Xylazine-induced extracellular Ca^{2+}-dependent contractions were lower in amplitude but longer in duration than those elicited by noradrenaline or phenylephrine (Figs 1, 3).

Influence of prazosin and yohimbine on noradrenaline- and xylazine-induced cellular and extracellular Ca^{2+}-dependent contractions

Prazosin (0·03–0·3 μM) reduced the amplitude of both cellular and extracellular Ca^{2+}-dependent noradrenaline (5 μM)- and xylazine (1 mM)-induced contractions (n = 6, Fig. 4). However, prazosin was about ten times more effective in antagonizing cellular than extracellular Ca^{2+}-dependent xylazine-induced contractions (Fig. 4).

Yohimbine in concentration up to 3 μM did not reduce the amplitude of either cellular or extracellular Ca^{2+}-dependent noradrenaline (5 μM)- and xylazine (1mM)-induced contractions (n = 6).

Effect of xylazine on field stimulation induced contraction

Xylazine reduced contractions induced by supramaximal field stimulation in a concentration related manner, its ED50 and relative 95% confidence limits being 37·1 μM (18–76 μM; n = 6).

Effect of 6-hydroxydopamine pretreatment on cellular and extracellular Ca^{2+}-dependent noradrenaline and xylazine-induced contractions

Experiments were carried out to determine whether noradrenaline and xylazine effects were attributable to mechanism(s) other than a direct action on

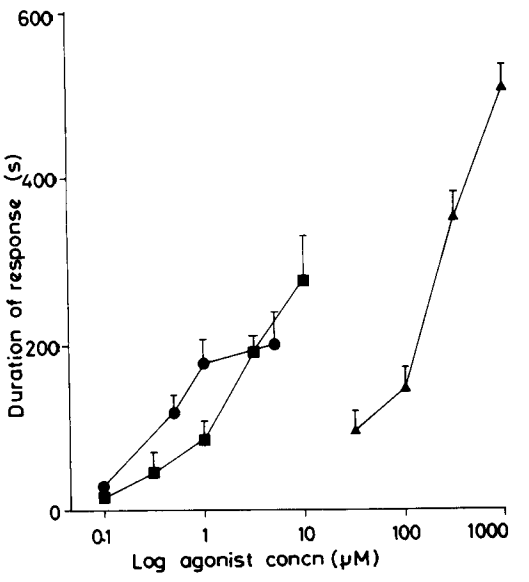

FIG. 3. Concentration-response curves for the duration of extracellular Ca^{2+}-dependent contraction induced by noradrenaline (●), phenylephrine (■) and xylazine (▲). Each point is the mean ± s.e. of at least six experiments.

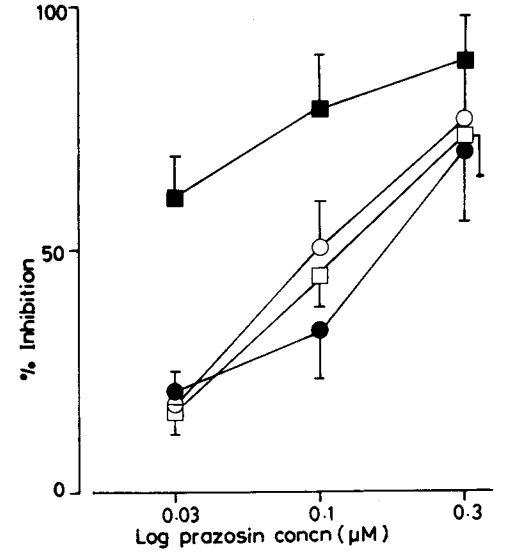

FIG. 4. Concentration response curves for the inhibition by prazosin of cellular (filled symbols) and extracellular (open symbols) Ca^{2+}-dependent contractions induced by noradrenaline (5 μM) (circles) and xylazine (1 mM) (squares). Each point is the mean ± s.e. of at least 5 experiments.

postjunctional α-adrenoceptors. After 6-hydroxydopamine pretreatment, concentration–response curves of cellular and extracellular Ca^{2+}-dependent xylazine-induced contractions were markedly shifted to the right. Maximal contractile values were also markedly reduced (Fig. 5).

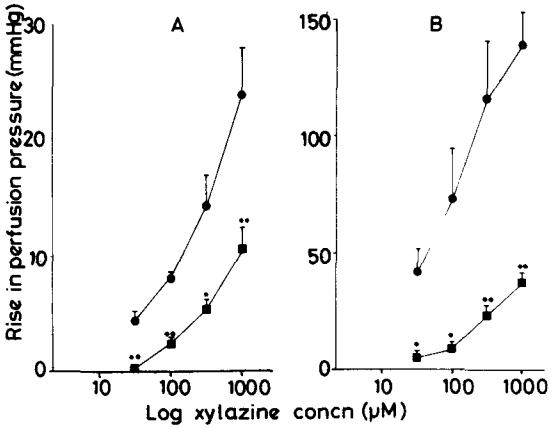

FIG. 5. Concentration-response curves for the amplitude of cellular (Panel A) and extracellular (Panel B) Ca^{2+}-dependent contractions induced by xylazine in control (●) and in 6-hydroxydopamine pretreated (■) arteries. Each point is the mean ± s.e. of 4 experiments.

On the other hand concentration-response curves of cellular and extracellular Ca^{2+}-dependent noradrenaline-induced contractions were barely affected by 6-hydroxydopamine pretreatment (Fig. 6).

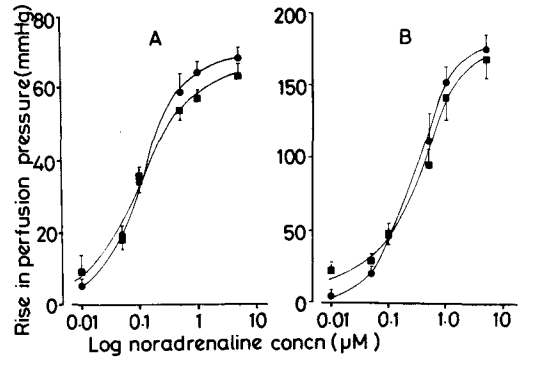

FIG. 6. Concentration-response curves for the amplitude of cellular (panel A) and extracellular (panel B) Ca^{2+}-dependent contractions induced by noradrenaline in control (●) and in 6-hydroxydopamine pretreated (■) arteries. Each point is the mean ± s.e. of experiments.

DISCUSSION

Our data indicate a qualitative and quantitative similarity between the capability of intraluminal noradrenaline and phenylephrine to mobilize both cellular and extracellular Ca^{2+} in rabbit ear artery. This strongly suggests that stimulation of α_1-adrenoceptors initiates a series of reactions leading to release of intracellular Ca^{2+} and opening of receptor-operated channels allowing extracellular Ca^{2+} entry. This suggestion is strengthened by biochemical findings showing that the metabolic effect of α_1-adrenoceptor stimulation is specifically linked to phosphatidylinositol turnover (Fain & Garcia Sàinz 1980). Phosphatidylinositol breakdown can determine both the release of bound intracellular Ca^{2+} as well as the entry of extracellular Ca^{2+} (Berridge 1980). Recently, a strict correlation between α_1-adrenoceptor stimulation and either phosphatidylinositol turnover or contraction, has been demonstrated in rabbit aorta (Villalobos-Molina et al 1982).

The fact that prazosin was equally effective in antagonizing both cellular and extracellular Ca^{2+} dependent noradrenaline-induced contractions whereas yohimbine has no such effects, further supports the hypothesis that α_1-adrenoceptor stimulation is responsible for the overall contractile activity induced by exogenous catecholamines. This agrees well with in-vitro findings indicating that noradrenaline-induced contraction of rabbit aorta and pulmonary artery (Docherty et al 1981; Docherty & Starke 1981) and canine femoral and splenic arteries (De Mey & Vanhoutte 1981) rely upon α_1-adrenoceptor stimulation.

The existence in rabbit ear artery of excitatory postjunctional α_2-adrenoceptors freely accessible to circulating catecholamines is not supported by our data. In fact, xylazine antagonized field stimulation-induced contraction, which shows that it has prejunctional α_2-adrenoceptor agonistic properties (Sullivan & Drew 1980), in doses 1/10–1/20th as high as those required to elicit cellular and extracellular Ca^{2+}-dependent contractions. This suggested that in our experimental conditions xylazine postjunctional agonistic properties depended, at least in part, upon involvement of α_1-adrenoceptors. This hypothesis could explain the powerful inhibition of its responses by prazosin, but not by yohimbine whereas its weak potency might be related to some sort of partial agonism, as suggested by Starke & Docherty (1982). However this hypothesis does not explain either the greater effectiveness of xylazine in inducing extracellular compared with cellular Ca^{2+}-dependent con-

tractions or the slow onset and longer duration of the former contraction. To determine whether or not this phenomenon was ascribable to some interference with neuronal release and/or reuptake of noradrenaline from nerve endings, experiments were carried out using 6-hydroxydopamine pretreatment to produce selective chemical sympathectomy (Aprigliano & Hermsmeyer 1976).

The use of tricyclic antidepressants, which to some extent possess α-adrenoceptor blocking properties (U'Prichard et al 1978), and cocaine, whose specificity to inhibit neuronal uptake in isolated blood vessels is questioned (Webb & Vanhoutte 1982), was avoided. 6-Hydroxydopamine reduced both types of xylazine-induced contractions (being more selective against those following the readmission of normal Krebs), while responses to noradrenaline were unaffected. These findings suggest that both types of xylazine-induced contraction, but to a larger extent the extracellular Ca^{2+}-dependent one, could be connected with a slow and long-lasting release of catecholamines. On the whole, the xylazine data argue against the presence of excitatory postjunctional α_2-adrenoceptors in the rabbit ear artery and cast some doubts on the use of xylazine as a selective α_2-adrenoceptor agonist.

In conclusion our findings support the hypothesis that stimulation of α_1-adrenoceptors mobilizes intracellular Ca^{2+} and opens sarcolemmal channels for transmembrane Ca^{2+} influx. Furthermore, in the rabbit ear artery, the overall contractile activity of exogeneous noradrenaline appears to rely exclusively upon stimulation of α_1-adrenoceptors.

REFERENCES

Aprigliano, O., Hermsmeyer, R. (1976) J. Pharmacol. Exp. Ther. 198: 568–577

Berridge, M. J. (1980) TIPS 1: 419–424

Bevan, J. A. Garstka, W., Su, C., Su, M. O. (1973) Eur. J. Pharmacol. 22: 47–53

De Mey, J., Vanhoutte, P. M. (1981) Circ. Res. 48: 875–884

Deth, R. C., Van Breemen, C. (1974) Pflügers Arch. 348: 13–22

Deth, R. C., Casteels, R. (1977) J. Gen. Physiol. 69: 401–416

Docherty, J. R. MacDonald, A., McGrath, J. C. (1979) Br. J. Pharmacol. 67: 421–422P

Docherty, J. R., Constantine, J. W., Starke, K. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. 317: 5–7

Docherty, J. R., Starke, K. (1981) J. Cardiovasc. Pharmacol. 3: 854–866

Drew, G. M., Whiting, S. B. (1979) Br. J. Pharmacol. 67: 207–215

Fain, J. N., Garcia-Sàinz, J. A. (1980) Life Sci. 26: 1183–1194

Hoffman, B. B., De Lean, A., Wood, C. L., Schocken, D. D., Lefkowitz, R. J. (1979) Ibid. 24: 1739–1746

Hunter, D. R., Haworth, R. A., Berkoff, H. A. (1982) Circ. Res. 51: 363–370

Karaki, H., Kubota, H., Urakawa, N. (1979) Eur. J. Pharmacol. 56: 237–245

Karaki, H., Weiss, G. B. (1980) J. Pharmacol. Exp. Ther. 213: 450-455

Langer, S. Z., Shepperson, N. B. (1982) TIPS 3: 440–443

Litchfield, J., Wilcoxon, F. (1949) J. Pharmacol. Exp. Ther. 96: 99–113

Manzini, S., Maggi, C. A., Meli, A. (1982a) J. Pharmacol. Meth. 8: 47–57

Manzini, S., Maggi, C. A. Meli, A. (1982b) J. Pharm. Pharmacol. 34: 195–196

McGrath, J. C. (1982) Biochem. Pharmacol. 31: 467–484

Robinson, C. P., Sastry, B. W. R. (1976) J. Pharmacol. Exp. Ther. 197: 57–65

Starke, K., Docherty, J. R. (1982) J. Cardiovasc. Pharmacol. 4: 53–57

Sullivan, A. T., Drew, G. W. (1980) Naunyn-Schmiedeberg's Arch. Pharmacol. 314: 249–258

Timmermans, P. B. M. W. M., Kwa, H. Y., Van Zwieten, P. A. (1979) Ibid. 310: 189–193

U'Prichard, D. C., Greenberg, D. A., Sheeham, P. P., Snyder, S. H. (1978) Science 199: 197–198

U'Prichard, D. C., Snyder, S. H. (1979) Life Sci. 24: 79–88

Villalobos-Molina, R., Hong, M. V. E., Garcia-Sàinz, J. A. (1982) J. Pharmacol. Exp. Ther. 222: 258–261

Webb, R. C., Vanhoutte, P. M. (1982) J. Pharm. Pharmacol. 34: 134–136