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On the importance of agonist concentration-gradients within isolated tissues. Increased maximal responses of rat vasa deferentia to (−)-noradrenaline after blockade of neuronal uptake

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It is assumed often that blockade of agonist uptake processes in isolated tissues results only in shifts to the left of the concentration-response curves to the agonist with no concomitant increase in the maximal response. This may not be true in tissues where diffusion is not fast enough to permit penetration of the agonist to a sufficient number of muscle cells for production of tissue maximal response. Under these circumstances an agonist-concentration gradient is created within the tissue which, when altered, could lead to an increase in the maximal response to the agonist. The increased maximal responses of rat vasa deferentia to (−)-noradrenaline after blockade of neuronal uptake by either cocaine or desmethylimipramine have been analysed in terms of a concentration-gradient hypothesis. The data are compared with a theoretical calculation based on a model of restricted diffusion of enzyme-substrates into structured tissues. Both the experimental data and theoretical calculations suggest that an altered concentration-gradient of (−)-noradrenaline within the muscle layers of rat vasa deferentia is responsible for the increased maximal response. The effects of such gradients are discussed in terms of quantitation of drug-receptor phenomena and as a caveat to ascribing increases in maximal responses to post-synaptic effects of uptake inhibitors.

Experiments with isolated tissues are invaluable to the process of drug-receptor classification because they allow control of muscle cell environment and drug concentration to such a degree that inferences can be made about drug-receptor interactions. Such studies often depend upon comparison of tissue responses to drugs before and after pharmacological intervention, a null method which assumes necessarily that the translation of the agonist-induced stimulus into observed response is unchanged throughout the course of the experiment. Theoretically, there is no reason for this always to be true; for example, alterations in agonist uptake properties of tissues can seriously distort observed responses (Furchgott 1972). Generally, it has been assumed that inhibition of uptake processes results only in a sinistral shift of concentration-response curves for agonists removed from the receptor compartment at a rate comparable to diffusion from the bath fluid. However, if the uptake process prevents activation of a sufficient fraction of the muscle cell populations for maximal tissue response, theoretically, inhibition of uptake can produce an increase in the maximal response to the agonist as well. The increased maximal responses of rat vasa deferentia to noradrenaline produced by cocaine (Kasuya & Goto 1968; Westfall & Fleming 1971; Lee et al 1975; Westfall 1977) suggested an example where agonist concentration-gradients within a tissue could affect observation of true maximal responses. In this paper the relationships between the neuronal uptake of noradrenaline, the free diffusion of this agonist into the tissue and the number of smooth muscle cells required for production of maximal responses will be examined. The results will be compared to a theoretical model of restricted diffusion based on a model of enzyme-substrate concentration gradients within structured tissues described by Green (1976).

MATERIALS AND METHODS

Pairs of vasa deferentia were removed from male rats (Sprague-Dawley, 350–400 g) killed by cervical dislocation. The tissues were desheathed, mounted longitudinally in 30 ml organ baths by attachment to a Perspex holder and a Grass FT 0·03 isometric transducer and incubated under 0·5 g resting tension in Krebs-Henseleit solution at 37 °C gassed with 95%–5% oxygen and carbon dioxide. Isometric contractions were recorded on a Beckman R-511A dynograph recorder. Tissues were exposed to alternate concentrations of (−)noradrenaline (NA) of 10 and 100 μM for 1 min every 20 min during the first 2 h of incubation by which time responses were stabilized and showed no significant differences over the remainder of the experiment (usually 2½ h). Concentration-response curves were obtained by addition of NA to the bath fluid (0·1 ml stock

solutions), observation of peak response and immediate washing every 8 min. Longer times between concentrations had no effect on responses. After control concentration-response curves to either (−)-noradrenaline or methoxamine had been determined, tissues were incubated with either cocaine (10 μM) or desmethylimipramine (DMI) (0·2 μM) for 1 h. Concentration-response curves were then repeated. Under the conditions used artifacts arising from desensitization were eliminated (Westfall & Fleming 1971).

Drugs and solutions

Tissues were incubated in Krebs-Henseleit solution containing (mM): Na^+ 151, K^+ 3·4, Ca^{2+} 2·5, Mg^{2+} 1·2, Cl^- 128·4, HCO_3^- 30, SO_4^{2-} 1·2, $H_2PO_4^-$ 1·0, D-glucose 5·5. (−)-Noradrenaline HCl (NA, Sigma), methoxamine HCl (Burroughs Wellcome), desmethylimipramine (DMI, CIBA-Geigy) and cocaine HCl (Mallinckrodt Co.) were prepared in 100 μM ascorbic acid and kept on ice.

RESULTS

Cocaine (10 μM) sensitized rat vasa deferentia to NA as shown by the shift to the left of the mean concentration-response curve (Fig. 1A). Also, the maximal responses to NA were increased by cocaine (control 2·4 g, after cocaine 3·6 g, n = 4, paired *t*-test, t = 5·0, $P < 0.025$). Cocaine had no effect on the mean concentration-response curve to methoxamine, an α-adrenoceptor agonist not subject to removal from the receptor-compartment by neuronal uptake (Trendelenburg et al 1970) (Fig. 1B).

Qualitatively similar effects were obtained with DMI, another inhibitor of neuronal uptake. Vasa deferentia were sensitized to NA and maximal responses to NA were increased (control 2·0 g, DMI 0·2 μM, 2·5 g, n = 4, paired *t*-test, $t = 3.2$, $P < 0.05$) (Fig. 2B). Quantitatively, the effects were not as large as with cocaine, possibly because of an α-adrenoceptor inhibition by this concentration of DMI (H. J. Leighton, personal communication). A three fold shift to the right of the mean concentration-response curve to methoxamine suggested this to be a significant factor (Fig. 2B).

These experiments suggested that neuronal uptake of NA was limiting its access to the complete mass of muscle cells producing contraction and as such resembled the occurrence of acetylcholine concentration gradients within ordered tissues containing cholinesterase in a structured matrix (Green 1976). This phenomenon has been modelled mathematically by Green (1976) from which the following equation has been adapted. The NA concentration gradient within the muscle mass of the vas deferens was calculated theoretically by:

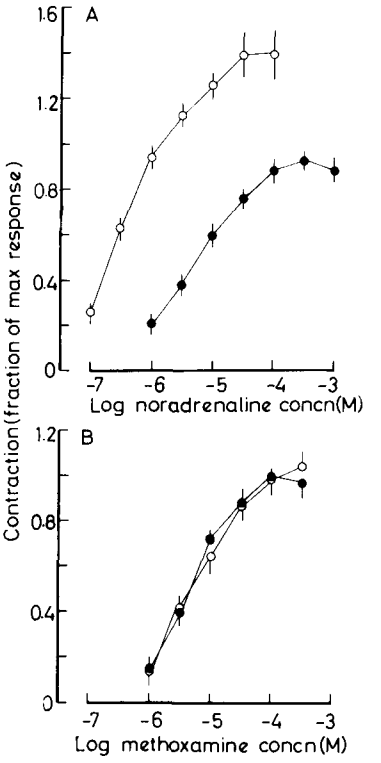

FIG. 1. Concentration-response curves of rat vasa deferentia. Contractions expressed as fractions of the maximal response to the agonist obtained in the absence of uptake inhibition. Abscissae: log concentrations of agonist (M). A: Responses to (−)-noradrenaline before (●) and after (○) cocaine (10 μM, 1 h). B: Responses to methoxamine before (●) and after (○) cocaine (10 μM, 1 h). Tissues were pairs from the same animal, n = 4. Bars represent standard errors of the means.

$$\frac{[NA]_r}{[NA]_o} = \frac{\cosh(\alpha r)}{\cosh(\alpha 1)}$$

$$\text{where } \alpha = \left(\frac{V_{max}}{K_m.D}\right)^{\frac{1}{2}}$$

The vas deferens was assumed to be a cylinder; 1 refers to the distance from the outer edge of the tissue to the centre axis and r refers to any point along 1 expressed as a fraction of 1 (Fig. 3A). $[NA]_r$ and $[NA]_o$ refer to the molar concentrations of NA at a depth r within the tissue and in the outside bathing medium respectively, K_m refers to the Michaelis-Menten constant for neuronal uptake and V_{max} the maximal rate of uptake. K_m and V_{max} were taken from studies on the uptake of [^{3}H]NA in rat heart

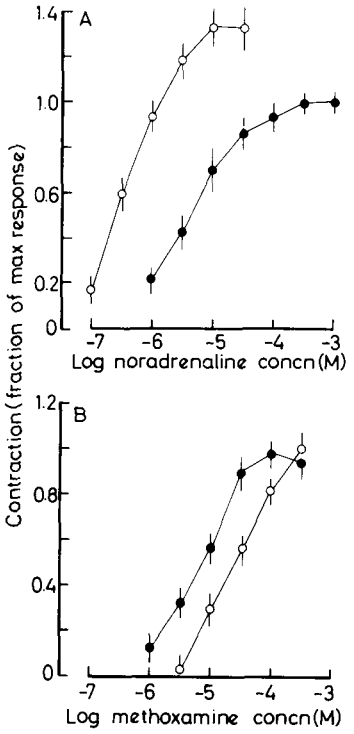

FIG. 2. Concentration-response curves of rat vasa deferentia. A: Responses to (−)-noradrenaline before (●) and after (○) DMI (0·2 μM, 1 h). B: Responses to methoxamine before (●) and after (○) DMI (0·2 μM, 1 h). Tissues were pairs from the same animal, n = 4. Bars represent standard errors of the means.

(Iversen 1967, $K_m = 0{\cdot}27\ \mu M$, $V_{max} = 0{\cdot}2\ \mu g\ min^{-1}\ g\ heart^{-1}$). In quantitative terms, it is a disadvantage to use the V_{max} obtained in rat heart as it may not be the same in rat vasa deferentia. However, a study with [^{3}H]NA in rat vasa deferentia demonstrated a neuronal uptake process with a calculated velocity of uptake approximately one-third to one half of the V_{max} in rat heart tissue at 0·7 μM noradrenaline (Birmingham & Iversen 1969). This suggests that the V_{max} in rat heart is not an unreasonable value to utilize in these calculations. Furthermore, theoretical calculations show that an unacceptably low V_{max} would have to be operative in rat vasa deferentia to make neuronal uptake incapable of producing a noradrenaline gradient within the muscle mass.

D refers to the diffusion coefficient of NA within the tissue, assumed here to be $10^{-5}\ cm^2\ s^{-1}$. This coefficient is for small ions (i.e. acetylcholine) in free solution and as such must be the upper limit for the rate of diffusion of NA into the tissue. In structured tissues such as the rat diaphragm, D has been

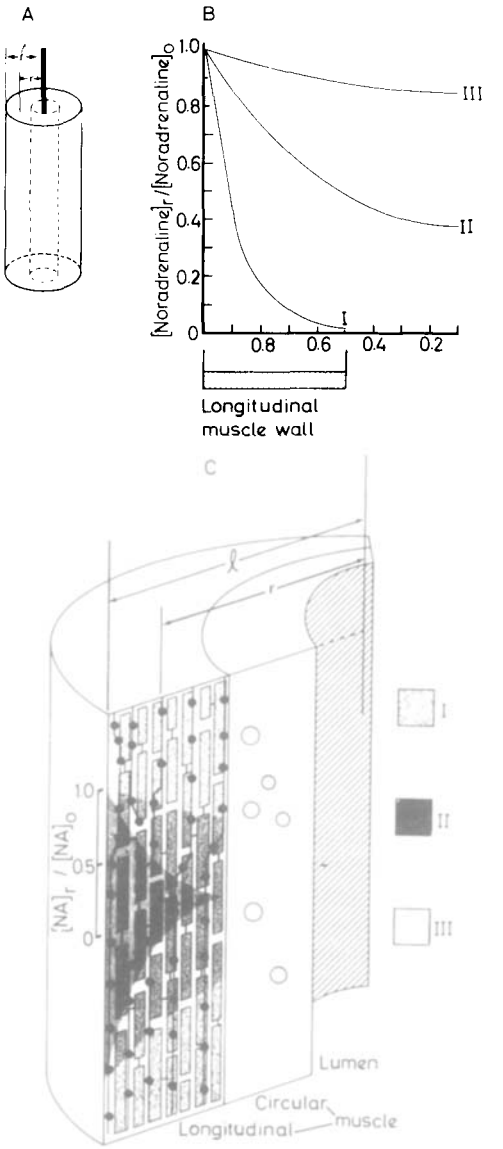

FIG. 3. A: Diagram depicting orientation of vasa deferentia with respect to l and r for theoretical calculation of gradients. B: Theoretical representation of (−)-noradrenaline concentration gradients in rat vasa deferentia. Ordinates: concentration of (−)-noradrenaline at a depth r within the tissue expressed as a fraction of the concentration in the bathing medium. Abscissae: distance from the outer edge of the tissue toward the centre axis expressed as a fraction of the total distance (l). I represents the gradient in the absence of uptake inhibition. II represents the gradient in the presence of cocaine (10 μM). III represents the gradient in the presence of DMI (0·2 μM). C: Schematic diagram of a cross-section of a rat vas deferens. Detail shows the concentration gradients in the absence and presence of cocaine (10 μM) within the outer wall of longitudinal smooth muscle cells. I longitudinal smooth muscle cell, II (−)-noradrenaline gradient in the absence and III, in the presence of cocaine.

estimated to be lower than 10^{-5} cm^2 s^{-1} (Krnjevic & Mitchell 1960: Brookes & Mackay 1971). Thus, the concentration-gradient calculated with this value of D is almost certainly underestimated.

In spite of the lack of quantitative parameters for this tissue, the calculation has descriptive value. Fig. 3B shows the calculated concentration-gradients for NA within the tissue in the absence and presence of cocaine (10 μM, where the K_I for cocaine has been assumed to be 0·34 μM; Iversen 1973) and DMI (0·2 μM, where the K_I has been assumed to be 8·9 nM; Iversen 1973). In the absence of uptake inhibition, theoretically only an outer layer of muscle cells is exposed to NA and the thickness of this layer is increased by inhibition of uptake (Fig. 3C).

DISCUSSION

The experimental data confirm the significant cocaine-induced increase in maximal-responses of rat vasa deferentia to noradrenaline found by other workers (Kasuya & Goto 1968; Westfall & Fleming 1971; Lee et al 1975; Westfall 1977). It has been suggested that the increased maximal response results from a post-synaptic potentiating property of cocaine due to a facilitation of calcium mobilization (Kasuya & Goto 1968; Greenberg & Long 1971). Although a significant post-synaptic action of cocaine has been described (Maxwell et al 1966), in this tissue it is unlikely to be the causative factor in the increases of maximal responses observed in our experiments. Cocaine did not increase the maximal responses to methoxamine, an α-adrenoceptor agonist which is not a substrate for neuronal uptake (Trendelenburg et al 1970) and the increased maximal response to NA was produced by DMI, another inhibitor of neuronal uptake. Both of these facts contribute to the cogency of the hypothesis that neuronal uptake of NA is the factor to account for this phenomenon. A hypothesis that relates the increased maximal response to active removal of noradrenaline from the receptor compartment has been proposed by Pennefather (1973). It states that the maximal response to noradrenaline is directly related to the rate of entry of noradrenaline into the tissue. This theory is predicated on the acceptance of rate theory of drug action (Paton 1961), a prerequisite that must subordinate it to hypotheses not requiring the assumption that rate theory governs activation of α-adrenoceptors by noradrenaline in this tissue.

The formation of noradrenaline concentration gradients within the tissue provides a simple hypothesis which can explain the experimental results, including those of Pennefather (1973), with minimal assumptions. This hypothesis requires two main assumptions. Firstly, neuronal uptake must remove NA from the receptor-compartment at a rate comparable to its entry into the muscle mass by diffusion. The theoretical calculation (Green 1976), suggests that neuronal uptake and diffusion would compete to produce a progressive fall in the NA concentration toward the central axis of the tissue. The possible overestimation of V_{max} is offset by the nearly certain overestimation of the rate of diffusion of NA to make the calculation valid at least qualitatively. An interesting parallel can be drawn between the predictions from this model of estimations of the potencies of enzyme inhibitors (expressed as K_I, the equilibrium dissociation constant of the inhibitor for the site of antagonism) and the inhibitor of neuronal uptake in rat vasa deferentia by cocaine. Values of K_I for enzyme-inhibitors derived from homogenized tissues are often much higher than their K_I values from structured isolated tissues containing the same enzyme (for examples see Green 1976). This is thought to be because the diffusion characteristics of the substrate into the tissue mass are determined by, among other things, the spatial arrangement of the enzyme within the tissue. Therefore, a concentration gradient of substrate may develop and only a fraction of the enzymic pool may be assayed at any one time. Inhibition of the enzyme alters this gradient, a larger proportion of the enzyme pool is assayed and an underestimation of the inhibition of the enzyme reaction results (Green 1976). A parallel can be drawn between these enzyme effects and neuronal uptake in rat vasa deferentia. Pertinent to this paper is the fact that the estimate of K_I for the inhibition of the neuronal uptake of [^{3}H]NA in intact rat vasa deferentia is 40 times greater than in rat perfused heart (vas deferens K_I = 14·1 μM, Drew et al 1978; rat heart, K_I estimated from IC50 of 0·38 μM = 0·34 μM) a fact possibly related to the noradrenaline concentration gradient in the vasa deferentia.

Similar concepts have been discussed by Wilson & Dietschy (1974) who, in studies of active transport processes in intestine, used mathematical models to describe artifically high K_m values for active transport resulting from an unstirred water layer, such as might be encountered in the muscle mass of vasa deferentia.

The rat vas deferens is densely innervated with neurons evenly distributed throughout the muscle cell layers (Zieher & Jaim-Etcheverry 1971; Anton et al 1977), and individual innervation of every cell by one or more close neuromuscular junctions

(200 Å) has been observed (Burnstock 1970). The relative geometry of uptake sites has been correlated with the magnitude of uptake effects (Trendelenburg 1972) thus the dense and close innervation of smooth muscle cells favours a significant removal of exogenous NA from the region of α-adrenoceptors.

The second assumption, required for the concentration-gradient hypothesis, is that the number of cells activated by NA, in the presence of active neuronal uptake, is insufficient to produce the maximal α-adrenoceptor mediated response of the tissue. The relationship between fractional activation of muscle mass by α-adrenoceptor agonists and observed response is unknown but there is morphological and pharmacological evidence to suggest that activation of an outer layer of smooth muscle cells would be insufficient to activate the whole tissue. Electrical coupling between smooth muscle cells via regions of close apposition of cell membranes termed nexuses (Dewey & Barr 1962) has been shown to be much less prevalent in densely innervated tissues like the rat vas deferens (Merrillees et al 1963) thus an α-adrenoceptor stimulus received by a small number of cells would be less readily transmitted throughout the tissue mass. Under physiological conditions, extensive cell to cell contact would not be needed as the dense innervation would be capable of producing fast synchronous activity. This tissue has been described as demonstrating multiunit behaviour (Prosser 1962; Jewell 1968) observed experimentally in double compartment bath experiments by Goto et al (1976). Vasa deferentia were incubated in a bath divided into two sealed compartments and addition of agonist to one compartment produced only partial contraction, indicating poor electrical coupling of smooth muscle cells.

Experiments with rat denervated vasa deferentia suggest further that the innervated vas deferens demonstrates incomplete recruitment of the smooth muscle cell populaion. It has been proposed that, after denervation, the increase in maximal responses to agonists is a direct result of increased synchronization of contraction by an improvement of cell to cell contact (Lee et al 1975; Westfall et al 1975; Kasuya & Suzuki 1978). This suggests that in innervated vasa deferentia the existing cell to cell contact is insufficient to produce true tissue maximal responses by activation of a fraction of the cell population.

It is pertinent to reconsider the findings of Pennefather (1973) that vasa deferentia desensitized to exogenously added noradrenaline produced a substantially larger maximal response to nerve stimulation when compared with the maximal response to exogenous noradrenaline. Such a result would be predicted if the exogenous noradrenaline penetrated to and desensitized the outer layers of muscle cells while the neuronally released noradrenaline, by virtue of the morphological location of the adrenergic neurons, activated inner cells not desensitized. Consistent with this hypothesis is the fact that cocaine produced an increase in the maximal response of exogenously added noradrenaline but not to nerve stimulation (Pennefather 1976). As with desensitization, the concentration gradient to exogenously added noradrenaline would be diminished by cocaine, leading to an increase in maximal response. No gradient being operative for neuronally released noradrenaline, only a sensitization, with no increase in maximal response, would be produced by cocaine.

The pharmacological and morphological data are consistent with the concentration gradient hypothesis for noradrenaline in this tissue. Gradients may be operative for a number of agonists for which there are degradative or uptake mechanisms in this tissue, since it is known that the maximal responses to acetylcholine, adrenaline and 5-hydroxytryptamine are different from each other and less than the response to barium chloride (Jurkiewicz et al 1969). Concentration-gradients have been proposed for noradrenaline in rabbit ear artery (De la Lande et al 1967) and rabbit aortic strip (Pascual & Bevan 1978). There are indications that the effect may become more important with increasing thickness of tissue. The effect of the COMT inhibitor U-0521 (3′4′-dihydroxy-2-methylpropiophenone) on increasing the maximal responses of dog saphenous vein to isoprenaline was more pronounced in thick rather than thin preparations (Guimãraes et al 1975). This concept has been quantitated in terms of volume/surface ratios of guinea-pig papillary muscles by Ebner & Waud (1978) indicating that uptake effects become more important in tissues with large volume/surface ratios.

The agonist concentration gradient hypothesis has implications for the quantitation of tissue sensitization after inhibition of agonist uptake processes. It has been suggested that inhibition of uptake theoretically should produce only a sinistral shift of concentration-response curves (designated as Type I sensitization by Kalsner 1974). However, this is true only if the number of cells activated before inhibition of uptake is sufficient to produce the maximal tissue response. Thus, an increase in maximal response should not automatically be taken as evidence of a post-synaptic effect.

In terms of quantitative experiments aimed at receptor classification alteration of agonist concentration-gradients can produce misleading information. For example, quantitative analysis of simple competitive antagonism depends upon parallel concentration-response curves with no alterations in maximum responses (Arunlakshana & Schild 1959). Thus, an antagonist with substantial uptake blocking properties theoretically could increase maximal responses, making analysis of antagonism difficult (for an example in rat vasa deferentia see Jurkiewicz & Jurkiewicz 1976). Further, an uptake blocking property of an antagonist, producing an increase in maximal responses, could mask a depressant or non-competitive receptor antagonism. In quantitating agonist activity, the comparison of α-adrenoceptor full and partial agonists on preparations such as rat vas deferens could lead to capricious results if the maximal responses were subject to tissue penetration and uptake effects; relative intrinsic efficacy comparisons would have little meaning.

In conclusion, although uptake effects have been known to produce artifacts in quantitative estimates of drug parameters (Furchgott 1972), such effects, when coupled to incomplete activation of muscle cell populations and concentration gradients within tissues, can provide distortion of maximal response measurements thereby confusing quantitation of competitive antagonism and intrinsic efficacy. Also, increases in maximal responses to agonists after blockade of uptake processes by an uptake inhibitor should not be taken as evidence of post-synaptic effects of the uptake inhibitor. It is possible that a substantial concentration-gradient of the agonist exists within the tissue and this possibility should be eliminated before new properties are ascribed to inhibitors of agonist uptake.

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