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by

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ABSTRACT

KINETIC CHARACTERIZATION OF THE RABBIT AORTA
RESPONSE TO ALPHA-1 ADRENERGIC AND SEROTONERGIC AGONISTS

by

Robert N. Cory

Advisers: Roman Osman Ph.D.
Saul Maayani Ph.D.

Both the alpha-1 adrenergic and serotonergic receptor mediated responses of the rabbit aorta were separated into a phasic and tonic response by virtue of their different dependence on extracellular calcium concentration. The kinetics of each response was characterized with respect to its dependence on both the concentration of drug and calcium. The phasic response is independent of extracellular calcium and has a rapid onset followed by a first order decay. Although its maximal attainable response is saturable with respect to the concentrations of drug and calcium, its rate constant for onset does not depend on the concentration of calcium in the preincubation buffer. This rate constant of onset is not saturable with respect to drug concentration suggesting that the rate - determining step of the phasic response is the diffusion controlled formation of the drug - receptor complex. The tonic response depends on extracellular calcium, shows first order kinetics of onset and reaches a steady state level of contraction that is sat-

urable with respect to the concentrations of drug and extracellular calcium. The rate constant for the generation of the tonic response depends on drug concentration in a saturable manner and linearly on extracellular calcium concentration. This suggests that the rate - determining step is the activation of a hypothetical effector by the drug - receptor complex. The activated effector would enable the transport of calcium ions into the cell. The kinetic studies show that the efficacy of a drug in this system is the maximal rate of activation of the effector by the drug - receptor complex. It was shown that this efficacy term depends explicitly on the total number of receptors and implicitly on the nature of the drug.

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3) Cory, R.N., Osman, R. and Maayani, S: A kinetic model for contractile responses of the rabbit aorta to agonists and partial agonists. Abstract, 67th Annual Meeting, Fed. Am. Soc. Exp. Biol., 1983.

ABBREVIATIONS

AMT	alpha - methyltryptamine
DMT	dimethyltryptamine
EPI	epinephrine
NMT	N - methyltryptamine
PE	phenylephrine
5-HT	5 - hydroxytryptamine
Quip	quipazine
Tryp	tryptamine
k_{int}	intrinsic rate constant of the tonic response
k_{obs}	observed rate constant of the tonic response
R_{eq}	equilibrium response level of the tonic response
k_{on}	rate constant of onset of the phasic response
k_{decay} ..	rate constant of decay of the phasic response
R_0	maximal obtainable respone of the phasic response

INTRODUCTION

The evolution of receptor theory is marked by attempts to formulate more precisely the relationship between the concentration of a drug and the response it elicits. Despite the progress in understanding receptor mediated effects there remains a large gap in the understanding of how receptors regulate post - receptor events in the response generating process. In part, this may be attributed to the lack of development of a proper methodology for studying receptor control over these events. To understand the mechanisms by which the receptor mediates the response it is necessary to measure the temporal behavior of the components that intercede between the initial drug - receptor interaction and the measurable response. Only recently have kinetic approaches been applied to study the mechanisms of pharmacological response generation. Levitzki et al. analyzed the kinetic properties of the beta - adrenergic linked adenylate cyclase in turkey erythrocytes (Tolkovsky and Levitzki, 1978; Tolkovsky, Braun and Levitzki, 1982). The kinetic analysis of response generation (cAMP accumulation) revealed the rate - determining step and yielded precise information about the time dependent interactions of the drug - receptor complex (DR) and the cellular components that lead to the observed response. Furthermore, the kinetic model of the response

provides an explicit description of the stimulus properties of a drug, (i.e., the drug's efficacy) in terms of the rate constants of the rate - determining step in response generation.

The kinetic analysis was made possible largely because many of the biochemical and functional characteristics of the response constituents had previously been elucidated by other investigators. However, this depth of understanding of the biochemical constituents of a receptor mediated response does not exist for most pharmacological preparations. In particular, there is a paucity of information about the initial steps in the generation of drug induced contractions in isolated smooth muscles, a mainstay tool of pharmacologists. Nevertheless, the kinetic analysis of the contractile response of an isolated smooth muscle preparation is potentially informative. The dependence of the rate - determining step on the concentration of the drug and other essential reagents may lead to a mechanistic hypothesis about the nature of the coupling between DR and a second messenger system in the response generating process. To evaluate the role of kinetic analysis of pharmacological responses it is proper to discuss it in the context of the development of receptor theory.

Evolution of Receptor Theory:

A) Occupancy Theory

Attempts to quantify the behavior of drugs at receptor sites were initiated by Clark (1933). He suggested that the combination of drug and receptor could be described by the laws of mass action which state that in a bimolecular reaction at equilibrium the ratio of the concentration of reactants to products is a constant. For the drug - receptor interaction the following scheme can be written:

$$(1) \qquad [D] + [R] \underset{}{\overset{K_D}{\rightleftharpoons}} [DR]$$

where,

[D] = the concentration of the drug

[R] = the receptor concentration

[DR] = the concentration of the drug - receptor complex.

and K_D, the equilibrium dissociation constant, is defined as:

$$(2) \qquad K_D = [D][R] / [DR]$$

Using the law of the conservation of mass the concentration of the total receptor population (R_{tot}) is equal to the sum of the concentrations of the free receptor (R) and the occupied receptor (DR);

(3) $$[R_{tot}] = [R] + [DR]$$

From equations (2) and (3) the following hyperbolic relationship between [DR] and [D] can be derived (Ariens, 1954):

(4) $$[DR] = [R_{tot}]\,[D] / ([D] + K_D)$$

The assumptions underlying this occupancy theory are: i) the response is a linear function of the number of receptors occupied by the drug,

(5) $$\text{Response} \propto [DR]$$

and ii) receptor activation by a drug persists as long as the receptor is occupied by the agonist. In this formulation occupation of 50% of the receptors leads to 50% of the maximal response. This occurs when $[D] = K_D$; therefore, K_D is equal to the concentration of drug that elicits half the maximal response (the EC_{50}).

The observation that not all drugs elicit the same maximal response in the same pharmacological preparation led Ariens (1954) to introduce a proportionality constant, ℓ, which defines the intrinsic activity of a drug:

(6) $$\%\ \text{Response} = \ell\,[DR] / R_{tot}$$

The intrinsic activity is an arbitrary constant expressing the ratio between the maximal response of any drug and that

of the agonist with the greatest maximal response. Consequently, all drugs with an intrinsic activity equal to 1 are called full agonists; drugs which do not elicit a maximal response have an intrinsic activity between 1 and 0, and antagonists have an intrinsic activity equal to 0.

The observation that elimination of a fraction of the receptors (e.g. by alkylation) did not diminish the maximal response led to further modifications in the occupancy theory. Stephenson (1956) proposed that the response is a monotonic function of a stimulus (S), which itself is linearly related to receptor occupation through an ad hoc parameter "e" called efficacy. Thus:

(7) $$\text{Response} = f(S) = f(e\,[D] / ([D] + K_D))$$

Efficacy is not equivalent to intrinsic activity. According to Ariens's formulation, drugs that have the same intrinsic activity should occupy the same fraction of receptors to elicit equal responses. This is in contradiction with experimental results. Intrinsic activity does not distinguish between drugs having the same maximal response but different stimulus properties. Efficacy, however, accounts for the fact that different occupancies by different drugs can produce the same response if they generate the same stimulus. Thus, a drug can exert a large stimulus even if $[DR]/[R_{tot}] \ll 1$, as long as it has a large efficacy, i.e., occupation of a small fraction of the

receptors can elicit a maximal response. The concept of "spare receptors" arises from this theory.

The efficacy parameter as defined by Stephenson is not independent of the tissue. This can be demonstrated by the fact that the same drug may have different efficacies at the same receptor in different tissues. Furchgott and Burstyn (1967) suggested that these differences arise from the different number of receptors in different tissues. In their modified formulation, the intrinsic efficacy (ϵ) is a property of the drug alone and it relates to Stephenson's efficacy in the following manner:

(8) $$\epsilon = e / [R_{tot}]$$

Thus, the formulation of the relationship between occupancy and response becomes:

(9) $$\text{Response} = f(S) = f(\epsilon [DR]) = f(\epsilon R_{tot} [D]/([D] + K_D))$$

The drug dependent factors are ϵ and K_D, the tissue dependent factors are R_{tot} and the functional dependence of response on stimulus.

Each stage in the development of occupancy theory yielded a more accurate description of the concentration - response relationship. The concept of spare receptors and its relationship to receptor occupancy and consequently to efficacy was an attempt to introduce a mechanistic approach

to understanding drug action. Yet this approach lacks an explicit description of the functional relationship between stimulus and response and therefore efficacy remains an ad hoc parameter (see equation 9).

B) Sequential Models of Drug - Receptor Elicited Responses

Black and Leff (1983) introduced a model of the relationship between receptor occupancy and the generation of a pharmacological response from which emerges an explicit definition of efficacy. Operationally, this model can be derived from two hyperbolic equations (see also Furchgott, 1964). The first equation describes the formation of the drug - receptor complex and is equivalent to equation 4. The second equation describes the dependence of the response (Resp) on the concentration of the drug - receptor complex (DR):

$$(10) \qquad Resp = Resp_{max} [DR] / (K_E + [DR])$$

where $Resp_{max}$ is the maximal response possible in this tissue and K_E is the concentration of DR that elicits half the maximal response. Combining the two hyperbolic equations, 4 and 10, yields the following hyperbolic relationship:

$$(11) \qquad Resp / Resp_{max} = [R_{tot}] [D] / (K_D K_E + ([R_{tot}] + K_E) [D])$$

Three essential features of agonism are inherent in equation 11: K_D - the drug affinity for the receptor, R_{tot} - the total receptor number, and K_E - the efficacy of agonism. Black and Leff define the transducer ratio "tau" (τ) as $[R_{tot}] / K_E$. This ratio is a measurement of the "efficiency of transduction of occupied receptors .. into pharmacological effect". Equation 11 can now be written:

$$(12) \quad Resp/Resp_{max} = \tau [D] / (K_D + (\tau + 1) [D])$$

Tau depends on both K_E and R_{tot} and is analogous to Stephenson's efficacy term. It describes the translation of stimulus into response and is not purely a drug or a tissue dependent parameter.

The operational model (equation 11) can be represented by a system in which the response is produced by a coupling of the receptor to an effector (E):

$$(13) \quad D + R \underset{}{\overset{K_D}{\rightleftharpoons}} DR + E \overset{K_E}{\rightleftharpoons} DRE \longrightarrow \text{Response}$$

This is the simplest sequential model in which a drug - receptor complex (DR) interacts with an effector to form the activated effector, the ternary complex (DRE). In this model the concentration of DRE determines the magnitude of the ensuing response. It was shown (Black and Leff, 1983) that under certain conditions this system can be described by equation 11. It immediately follows that the efficacy of the drug is reflected in the dissociation constant K_E which is an inverse measure of the affinity of DR for E. De Lean et al.'s (1980) analysis of the beta - adrenergic linked cyclase of frog erythrocyte is in full agreement with this model. It should be noted that the ternary complex model is analagous to the floating receptor model introduced by Jacobs and Cuatrecasas (1974a, 1974b, 1976) and described by Boyenaens et al. (1975). In the floating receptor model the receptor is mobile in the cell membrane and so may interact with a number of effectors. Although the mathematical description of the model as presented by Boyenaens et al. (1975) is more complete than that of the ternary complex model both the floating receptor and ternary complex models

are described operationally by the formulation of Black and Leff (1983) (see above). These models, in which the generation of the response involves a sequence of coupled events, introduce two novel concepts into receptor theory: i) an event following the formation of DR but prior to response generation regulates the response level; in the Stephenson model DR alone regulates the response so the role of an effector is not acknowledged, and ii) the drug exerts its effect by modulating the binding of R to E, thus the affinity of DR for E is a measure of the efficacy of the drug; in Stephenson's model efficacy is an ad hoc parameter that cannot be attributed to a physical property of the system.

A Role for Kinetic Analysis of Pharmacological Systems:

The sequential models discussed above provide a basis for studying the coupled events which underlie pharmacological responses. In one aspect however, these models are deficient; the temporal nature of the coupling events is ignored. The intermediates that lead to response formation (e.g. DR and DRE) may not achieve a steady state concentration but rather may be in a constant state of flux. The importance of the time dependent properties of the response is illustrated in the following example. Consider the sequence of events:

$$(14) \quad D + R \xrightarrow{1} DR + E \xrightarrow{2} DRE \xrightarrow{3} \text{Response}$$

This is the same scheme as in equation 13 without the assumption that steps 1 and 2 reach equilibrium before the response is generated in step 3. There are at least three ways in which this sequence of events may behave kinetically, (the arrows depict net fluxes rather than individual forward or reverse reactions): i) Assume step 1 is the slowest and is therefore rate - determining in the reaction; steps 2 and 3 occur much more rapidly so the rate of generation of the response is determined by step 1 and the magnitude of the response by the concentration of DR. The kinetics will be governed by the diffusion of D to R. ii) Assume step 3 is rate - determining, in this case DRE will be rapidly formed from DR. The rate of generation of the response will be determined by step 3 and the magnitude of the response, by the concentration of DRE. This is a "rapid equilibrium system" analogous to the sequential models described above. In a rapid equilibium model the kinetics of the coupling steps cannot be studied and therefore efficacy is expressed as the equilibrium constant of step 2 (the inverse of K_E in equation 11). iii) The third possibilty is that step 2 is rate - determining. Under these circumstances the rate of accumulation of DRE determines the rate of generation of the response. This is

called a "slow activation system" because the rate of response generation is commensurate with the rate of effector activation. Consequently, the efficacy parameter in this model is the rate constant of step 2, the rate - determining step. This rate constant is a measure of the drug - dependent efficiency of coupling between DR and E.

Tolkovsky and Levitzki (1978, 1981, 1982) conducted a series of experiments to characterize the kinetic properties of the beta - adrenergic linked adenylate cyclase in turkey erythrocyte. They observed that the accumulation of cAMP exhibits a lag time that decreases with increasing agonist concentration. At longer time periods the rate of accumulation was the same regardless of the agonist concentration. Levitzki and Tolkovsky showed that the kinetic equations that describe the rapid equilibrium model predict a linear accumulation of cAMP at all drug concentrations whereas the equations of the slow activation model predict that the response will accumulate with the observed concentration - dependent lag time. Therefore, the concentration - dependent lag time suggests that the slow activation of an effector (DRE) is the rate - determining step in cAMP accumulation. A kinetic analysis of the response data (obtained by integrating the cAMP accumulation curves) revealed that the observed rate constant of onset of the response (k_{obs}) exhibited a first order dependence on agonist concentration and reached a limiting value at

increasing agonist concentrations. The value of k_{obs} for a particular agonist concentration was decreased after a reduction of R_{tot} with an irreversible antagonist of the beta - adrenergic receptor. In fact, they found a linear relationship between R_{tot} and kobs. The saturable dependence of k_{obs} on the concentration of drug and its dependence on the absolute number of receptors indicated that the rate of generation of the response is dependent on a receptor mediated event (in this model the formation of DRE from DR).

From these studies evolved the collision coupling model of cyclase activation (for a review see, Tolkovsky, 1983). In the collision coupling model, which is a derivative of the slow activation model discussed above, DR and E "collide" to form DRE which then immediately converts into DRE´ (DR and E are bound to each other only for an instant, hence the term " collison coupling"). DRE then dissociates to DR and E´, which is the active form of the cyclase:

$$\underset{(15)}{} \quad \overset{1}{D + R \longrightarrow} \; DR + E \overset{2}{\longrightarrow} (DRE \overset{3}{\longrightarrow} DRE') \overset{4}{\longrightarrow} DR + E' \longrightarrow \text{Response}$$

Still, step 2 is the rate - determining step followed by a very rapid step 3, so DRE´ never accumulates. In this kinetic sequential model the efficacy of the drug is a measurable property of the system; it is the maximal

observed rate constant of the activation of the effector by DR.

The prior work on the concentration of adenylate cyclase by other investigators enabled Levitzki and Tolkovsky to carry out their detailed analysis of the turkey erythrocyte system. This in depth approach cannot easily be extended to other systems about which little is known of the effector mechanisms. Nevertheless a kinetic description of any response mechanism is more informative than the corresponding steady state description. In fact the kinetic analysis at long times (i.e. at equilibrium or steady state) reduces to the steady state description and is therefore inclusive of it. More importantly, a kinetic analysis describes the dependence of the rate - determining step on the concentration of drug and receptor. This approach elucidates the relationship between the various response components and allows the formulation of a mechanistic hypothesis to describe the response generating process.

Kinetics of Pharmacological Responses in Isolated Organ Preparations:

The first attempt at formulating a theory of the kinetics of drug action in an isolated organ preparation was introduced by Paton (1961) in his rate theory. The rate theory attempted to explain phenomena associated with receptor mediated responses which could not be accounted for by the occupancy theory. The usefulness of this theory is criticized because it could be confirmed only in the guinea pig ileum (Waud, 1968). Another difficulty with Paton's approach is its reliance on the measurement of antagonist kinetics, the extension of the theory to agonist kinetics is inferential. Paton relied on antagonists to avoid the post - receptor properties inherent in the agonist response. In using this approach he assumed that the rate - determining step in the response is the interaction of D and R. This model of response generation does not consider post - receptor coupling events that may determine the rate of the onset of the pharmacological response.

The early work on the kinetics of agonist - induced responses considered only the simplest model for response generation. In this model the drug - receptor interaction is rate - determining in the generation of the response. In a rapidly responding tissue this may not be an unreasonable assumption (Van Ginneken, 1977). In slower responding

tissues, e.g. the frog rectus abdominus or the rabbit aorta, since the rate of formation of the drug - receptor complex is probably diffusion - limited and therefore much faster than the rate of onset of the response, a post - receptor coupling event could be rate - determining. Characterization of the dependence of the rate of onset of the response on the concentration of agonist may reveal whether or not the rate reflects a diffusional or post - receptor event.

Bieger et al. (1970) were the first to propose a kinetic model of agonist - induced responses in an isolated organ preparation. They studied the kinetics of muscarinic agonist - induced negative inotropic responses in the guinea pig atrium and found that the response could be modeled according to a simple bimolecular mechanism:

$$D + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR \qquad (16)$$

Bieger's group and others (Jung et al., 1971; Lass et al., 1979) showed that the association rate constant (k_1) obtained from the kinetic data for a group of agonists varied inversely with the agonists' EC_{50} values while the dissociation rate constant (k_2) was invariant. Talitman (1976) and van Ginneken (1977) observed the same phenomenon using agonists that contract the isolated guinea pig ileum.

Talitman (1976) suggested that these results indicate a dependence of agonist efficacy on the agonist rate constant of association with the receptor. Antagonist potency, on the other hand, was said to depend on the rate constants of both association and dissociation. A shortcoming of the approach taken by these investigators is their assumption that the rate - determining step in the response is the formation of the drug - receptor complex. Consequently, efficacy is not a measurable property in their model. Our current knowledge of pharmacological responses indicates that many responses involve the interaction of a receptor with an effector that produces a second messenger; this step should be included in a kinetic analysis of the response.

A more comprehensive approach to modeling a pharmacological response was adopted by Triggle and Chang (1973). They proposed a model of the cholinergic response of the guinea pig ileum that takes into account the drug - receptor interaction and some post - receptor coupling events. The response generating process consists of three basic steps; 1) the drug - receptor interaction, 2) the induction of calcium translocation from outside the cell to inside, concomittant with a release of bound intracellular calcium, and 3) the interaction of intracellular calcium with the contractile proteins. The rate - determining step in this model is the translocation of calcium across the membrane. Triggle and Chang (1973) suggested that the

intrinsic activity of a drug is determined by the ability of DR to stimulate calcium translocation (they should actually have referred to the drug's efficacy rather than its intrinsic activity when discussing the efficiency of transduction of a stimulus). Triggle and Chang noted that the rate of onset of the response was dependent on the concentration of the drug and its nature, i.e. partial agonists have a slower rate of onset than do equiactive concentrations of full agonist (this observation was strictly qualitative and did not account for the effect of different concentrations of drug on the response rates). They observed a biphasic contractile response with a phasic and tonic component and noted that the two components have a different dependence on extracellular calcium concentration, however, no attempt was made to analyze the two response components separately. Consequently, their discussion of the response kinetics was limited only to a descriptive level. This descriptive approach was inevitable because the behavior of the components of the proposed model was formulated with equations that describe the system at steady state. The model, therefore, could predict some steady state properties of the system, i.e. the shape of the concentration - response curves, but could not be used to estimate the kinetic parameters of the response.

The discussion of the above work reveals a need for a more rigorous approach to the study of the kinetics of

agonist - induced responses. This work should be predicated on the accurate measurement of the kinetics of the response and its dependence on the concentration of agonist, the nature of the agonist, the number of receptors, and, if possible, on the concentration of the effector that participates in response generation.

THE PRESENT INVESTIGATION

The rabbit thoracic aorta was selected as the experimental preparation because of the considerable background information available on the physiology (see Appendix) and pharmacology of this tissue. The pharmacologically elicited contractions of the rabbit aorta are relatively slow (with an onset time of 1 to 40 min) and therefore are easy to record and analyze. In this investigation the kinetics of the contractions to agonists acting at either the alpha-1 adrenergic or the serotonergic systems were characterized. I will briefly review the pharmacology of these two receptor systems in the rabbit aorta.

Alpha - Adrenergic Receptor Classification:

The rabbit aorta possesses alpha - adrenergic receptors, which mediate contraction, and beta - adrenergic receptors, which relax a contracted tissue (Besse and Furchgott, 1976). In the course of studying alpha - adrenergic mediated contractions the beta - adrenergic relaxation response is routinely blocked with a micromolar concentration of the beta selective antagonist propranolol (Besse and Furchgott, 1976).

The alpha-1 selective antagonist, prazosin, has a high affinity (K_D approx. equal to 1 nM) for the alpha - adrenergic receptor in the rabbit aorta when measured either in contracting tissues by the method of Schild (1947) or by radioligand binding of [^{3}H]-prazosin (Awad et al., 1983). In contrast the alpha-2 selective antagonist, yohimbine, exhibits a low affinity (K_D approx. equal to 3 uM) for these receptors when measured in either the contracting tissue (Furchgott, 1955; Awad et al., 1983) or by radioligand binding of [^{3}H]-yohimbine (Tsai and Lefkowitz, 1978). Yohimbine has a much higher affinity (K_D approx. equal to 10 nM) for receptor systems which are believed to be predominantly alpha-2 adrenergic (Ruffolo et al., 1981).

An investigation into the role of alpha-1 receptors in mediating both ^{45}Ca mobilization and smooth muscle contraction in the rabbit aorta revealed that agonist - induced calcium uptake, calcium release, and muscle contraction were all sensitive to inhibition by 0.1 uM prazosin but not 1 uM yohimbine. The results of these studies with antagonists and of the rank order of agonist potencies (Sheys and Green, 1972, Besse and Furchgott, 1976; Docherty and Starke, 1981; 1982) indicate that the alpha-1 and not the alpha-2 receptor mediates contraction in the rabbit aorta.

Serotonergic Receptor Classification:

Two classes of serotonin receptors were proposed by Gaddum and Picarelli (1957). They identified D receptors (sensitive to dibenzyline blockade) on the smooth muscle of the guinea pig ileum and M receptors (sensitive to morphine antagonism) on the postganglionic nerves of Auerbach's plexus. The serotonin receptor in the rabbit aorta was first classified by Wurzel (1966) as a D receptor according to this classification scheme. But this scheme is inadequate, neither morphine nor dibenzyline are specific antagonists of the 5-HT receptor. As a consequence the D and M receptors of the guinea pig ileum were not unequivocally characterized.

A recently adapted classification scheme for 5-HT receptors is based on the identification of two types of 5-HT binding sites (5-HT_1 and 5-HT_2) in rat brain particulate fractions (Peroutka and Snyder, 1979; Leysen, 1982). The 5-HT receptor mediating contraction of the rabbit aorta is of a single class (Stollack and Furchgott, 1983) and appears to be a functional correlate of the 5-HT_2 binding site (Humphrey et al., 1982; Maayani et al., 1984). The binding site and the aorta receptor have a similar high affinity for several 5-HT competitive antagonists including: ketanserin, methysergide and spiperone (Apperley et al., 1976; Humphrey et al., 1982).

The 5-HT and PE mediated contractile responses in the rabbit aorta exhibit both a similar kinetic profile and a similar dependence on extracellular calcium (see Results). These similarities suggest that these receptors mediate their responses through a common calcium - dependent mechanism. There is strong evidence that like the adrenergic receptor, the serotonin receptor in vascular tissue is linked to the influx of extracellular calcium (Ratz and Flaim, 1984). Furthermore, there is evidence that in some systems, e.g. blowfly salivary gland (Berridge 1983) and rat cerebral cortex (Conn and Sanders Bush, 1984) the $5\text{-}HT_2$ receptor may stimulate the turnover of phosphatidylinositol (PI). Several studies suggest that there is a functional dependence of receptor mediated calcium flux on PI turnover (see Appendix).

I report here my investigations on the kinetics of the adrenergic and serotonergic induced contractons of the isolated rabbit aorta. The contractions studied here are biphasic with an initial phasic component followed by a tonic component (Bohr, 1963). In smooth muscles in which receptors mediating contraction are coupled to calcium fluxes, the phasic component seems to be due to the release of intracellular calcium, whereas the tonic component is generated by the influx of extracellular calcium (for

reviews see: Daniel et al., 1979; van Breemen et al., 1982, and the Appendix of this manuscript). I adopted a phenomenological approach in which the major rate - determining steps in the formation of the response were characterized in terms of their dependence on the concentration of agonist and calcium ions. As I demonstrate here, the properties of the kinetic constants of the rate - determining steps are related to the pharmacological concept of efficacy. Furthermore, the kinetic analysis of the receptor mediated response of an isolated organ preparation can be used to investigate mechanistic aspects of response generation.

MATERIALS and METHODS

Tissue Preparation:

Male New Zealand White Rabbits (Perfection Breeders, Douglassville, Pa.) 5 to 6 weeks old were killed by CO_2 asphyxiation and the thoracic aortae were rapidly excised. The adventitia were removed by the method of Maxwell et al. (1968) and the tissue was cut into rings 0.5 to 0.6 cm wide. The aortic rings were suspended between wire hooks in a 20-ml organ bath, at 36 $\pm$ 1^{o} C containing Krebs - bicarbonate buffer bubbled with 95% O_2 - 5% CO_2 to maintain a pH of 7.6 $\pm$ 0.2. The tissues were initially set at 3 g tension and allowed to relax over a period of 2.5 hr according to the procedure of Stollak and Furchgott (1983). When the resting tension of the tissues stabilized (at about 1.5 g), contractile responses, produced by adding 20 to 60 ul of a concentrated solution of drug to the bath, were measured isometrically with a Grass Force Displacement Transducer, model FT-03 connected to a Grass Polygraph, model 7D. Drugs were removed from the bath by washing out and replacing the buffer.

Solutions:

The normal Krebs - bicarbonate buffer contained (in millimolars) in distilled deionized water: NaCl, 124; KCl, 5; $MgSO_4$, 1.3; $CaCl_2$, 2.25; NaH_2PO_4, 0.6; $NaHCO_3$, 2.5; and glucose, 10. A calcium - free buffer was prepared by excluding $CaCl_2$ from the Krebs- bicarbonate buffer. The small contribution of $CaCl_2$ to the total chloride concentration and the osmolarity of the calcium - free buffer was negligible and, therefore, was not corrected for.

In all experiments 0.03 mM disodium EDTA was added to the buffer to reduce amine oxidation (Besse and Furchgott, 1976). The free calcium concentration in the buffer was corrected for the chelating effect of EDTA by taking into account the dissociation constant of Ca++ - EDTA and the competitive binding of magnesium to EDTA (Metzler, 1977). It is common practice in experiments with the rabbit aorta to use cocaine to block neuronal uptake of catecholamines (Besse and Furchgott, 1976; Docherty and Starke, 1981). In the experiments with PE, cocaine was not used because, in agreement with the findings of Bevan and Verity (1967), we found the effect of 30 uM cocaine to be negligible in the adventitia - free aorta preparation. PE had no apparent activity at the beta adrenergic receptors as evidenced by the lack of effect of 3 uM propranolol on the PE - induced response (not shown). In a few experiments with epinephrine

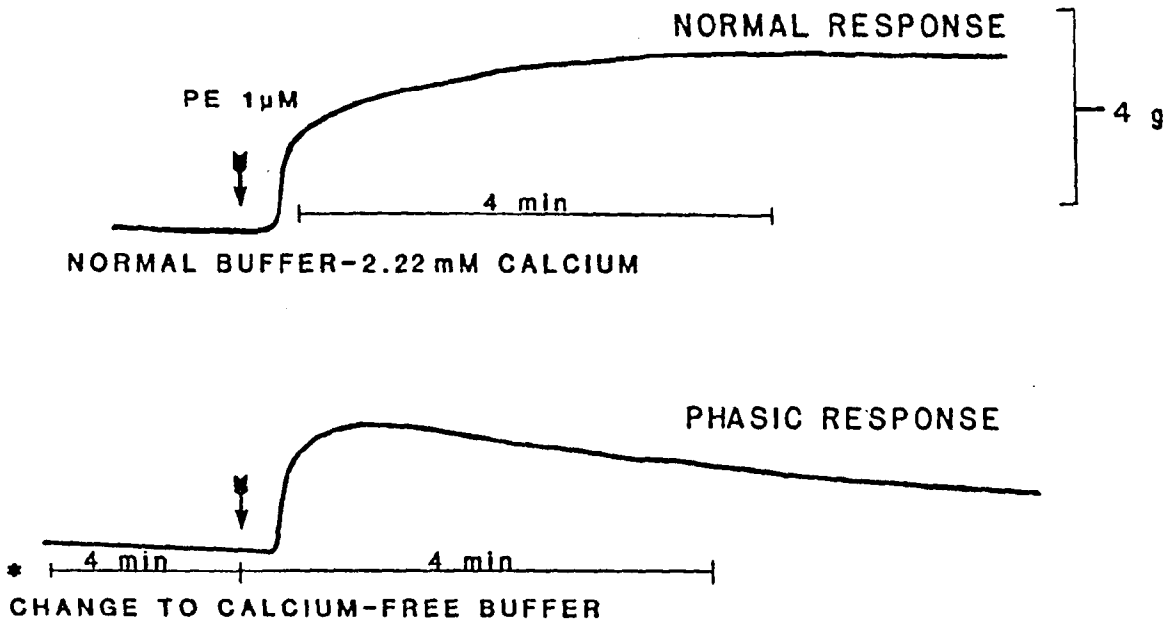
(EPI), 3 uM propranolol was used to block the beta adrenergic receptor and 30 uM cocaine to block neuronal uptake of EPI. Stollak (1980) showed that an extraneuronal uptake blocker is not necessary in this preparation.

In the experiments with 5-HT and the tryptamine analogs the aortic rings were pretreated with 720 uM iproniazid phosphate for 30 min to inactivate monoamine oxidase and with 15 uM benetrexamine tetrahydrocholoride monohydrate for 15 min to inactivate adrenergic receptors which could otherwise contribute to the tryptamine contractile response (Stollak and Furchgott, 1983). Cocaine was added to allow comparison of these studies with others on the 5-HT receptor (Clancy et al., 1982, Stollak and Furchgott, 1983).

Measurement of the phasic response:

The phasic response (fig. 1) was obtained by replacing the Krebs - bicarbonate buffer with a calcium - free buffer 3 to 4 min before adding the drug to elicit the contraction. To study the effect of calcium ion concentration on the kinetics of the phasic response of PE the tissue was preincubated for 30 to 40 min in the Krebs - bicarbonate buffer with a concentration of free calcium ranging from 0.12 to 2.22 mM; the phasic response was then generated as described above. This procedure is similar to the one em-

FIGURE 1: Tracing of an original polygraph recording demonstrating the method for eliciting the phasic response to PE. Three to four minutes before adding PE (arrow) to elicit a contraction, the Krebs - bicarbonate buffer was replaced (*) with a calcium - free buffer. Note the change in the time axis after the addition of PE.


NORMAL RESPONSE
PE 1μM
4 g
4 min
NORMAL BUFFER-2.22 mM CALCIUM
PHASIC RESPONSE
4 min
4 min
CHANGE TO CALCIUM-FREE BUFFER

ployed by Karaki et al. (1979) in their work on the phasic component of the rabbit aorta contractile response.

Measurement of the tonic response:

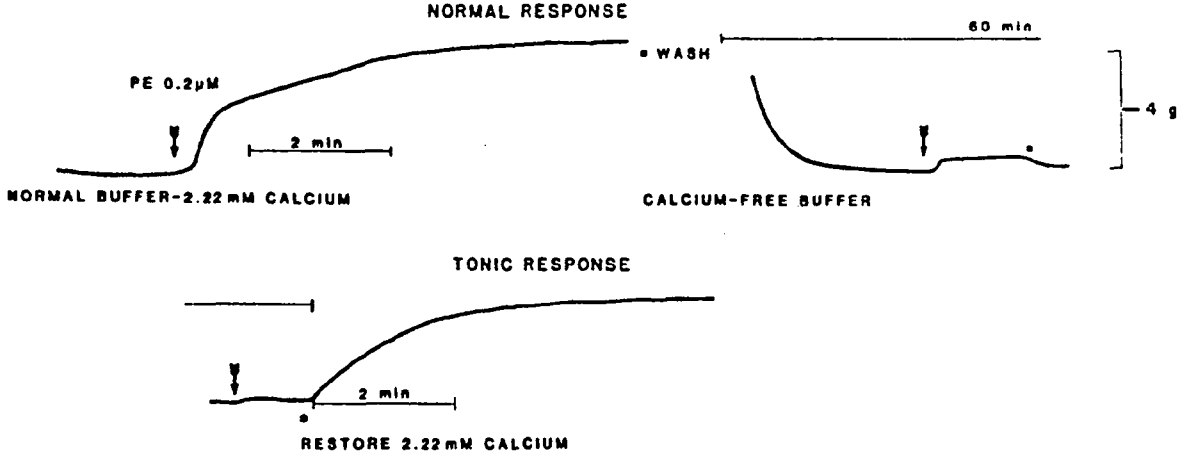
The ability of the tissue to produce the phasic response in the calcium - free buffer was exhausted by repeatedly stimulating it with drug. When a phasic response could no longer be elicited in the calcium - free buffer the tissue was incubated with drug for 10 to 60 sec after which time calcium was added. The addition of calcium at this point produced a tonic response (fig. 2) after a short lag period which was more evident at the lower concentrations of drug.

Data analysis:

Polygraph tracings of aorta contractions were digitized on the PROPHET Computer System. Each of the digitized points was assigned coordinate values that were converted to response levels as a function of time. The data were then used for graphical reconstructions of the response and for analysis of its kinetics. Estimates of EC_{50} values and slope indices (p) were obtained by fitting the equation:

(17) $$R = Rmax/(1 + (EC_{50}/[D])^{p})$$

FIGURE 2: Tracing of an original polygraph recording demonstrating the method for generating the tonic response to PE. Both curves shown are from the same tracing and have equivalent tension scales. Two times scales are shown, the faster during the generation of the response and the slower for the interval between the two responses. Arrows indicate additions of PE, the washout of the drug with the calcium - free buffer is indicated by a small square and the restoration of 2.22 mM calcium by an asterisk (*). See under "Methods" for a description of this technique.


NORMAL RESPONSE
PE 0.2μM
2 min
NORMAL BUFFER-2.22 mM CALCIUM
WASH
60 min
4 g
CALCIUM-FREE BUFFER
TONIC RESPONSE
2 min
RESTORE 2.22 mM CALCIUM

to the observed response (R) as a function of drug concentration [D] using either the nonlinear least squares fitting procedure FITFUN or LOGISTICS available on the PROPHET Computer System. FITFUN was also used to fit equation 21 (see under "Results") to the phasic response data.

The arithmetic mean and S.E. are reported for the average response of replicate experiments. For the average EC_{50} of replicate experiments the geometric mean and its S.E. are shown (De Lean et al., 1982). The number of replicate experiments (n) is signified in parentheses. Data from different rings within each experiment were pooled.

The efficacies of the partial agonists, DMT and quipazine, relative to the full agonist 5-HT were estimated using the method of Barlow et al. (1967) as modified by Kenakin and Black (1978). When equiactive concentrations of agonists and partial agonists are measured in the same tissue the following equation can be used to calculate the relative efficacy (e_P/e_A) of the partial agonist:

$$(18) \quad [A] = K_A e_P / (e_A - e_P) - [A] / [P] \cdot (K_P e_A / (e_A - e_P))$$

where

[A] = the equiactive concentration of full agonist

[P] = the equiactive concentration of partial agonist

K_A = the dissociation constant of the full agonist

K_P = the dissociation constant of the partial agonist

e_A = the efficacy of the full agonist

e_P = the efficacy of the partial agonist.

If the value of K_A is determined independently, for example, using the alkylation method described by Besse and Furchgott (1976) than equation 18 can be used to calculate directly K_P and the relative efficacy of the partial agonist (e_P/e_A). By plotting [A] vs. [A]/[P] the intercept (b):

(19) $$b = K_A\, u \,/\, (1 - u)$$

and the slope (m) are obtained:

(20) $$m = K_P \,/\, (1 - u)$$

where $u = e_P \,/\, e_A$.

With known values for b, m and K_A equations (19) and (20) can be solved simultaneously to find K_P and e_P/e_A.

Chemicals:

All drug solutions were prepared on the day of the experiment in glass distilled water and kept in an ice bath except solutions of dibenamine which were prepared immediately before use. The following drugs (abbreviations) were used: Phenylephrine HCl (PE), disodium EDTA, 5-hydroxytryptamine oxalate (5-HT), tryptamine HCl (Tryp),

N-methyltryptamine (NMT) [Sigma Chemical Co., St. Louis, MO.]; dl-alpha-methyltryptamine (AMT), benetrexamine tetrahydrochloride monohydrate, iproniazid phosphate [Aldrich Chemical Co., Milwaukee, WI.]; cocaine HCL, dimethyltryptamine (DMT) [NIDA]; dibenamine HCl [ICN Pharmaceuticals, Plainview, N.Y.] and 2-(1-piperazinyl) quinoline (quipazine maleate) (Quip) [Miles Laboratories Inc., Elkhart, Ind.]. All other compounds were of analytical grade.

RESULTS

PART I: KINETIC CHARACTERIZATION OF THE ALPHA-1 ADRENERGIC RESPONSE

The kinetic profile of the contractile response of a rabbit aorta ring to various concentrations of PE is shown in figure 3. The biphasic response, seen clearly at 100 uM PE, is composed of an initial phasic component followed by a tonic component. Consistent with observations by others (Brodie et al., 1959; Bohr, 1963; Deth and van Breemen, 1974, 1977) it was observed that the two components of the response were differentially sensitive to the level of extracellular calcium. By lowering the concentration of calcium in the Krebs - bicarbonate buffer 2 to 3 min before PE administration (fig. 4) the tonic component of the response can be gradually reduced without appreciably affecting the phasic component. After the phasic component reached its peak the response approached different steady - state levels depending on the calcium concentration in the buffer. When calcium was eliminated from the buffer, 3 to 4 min before PE administration, a contractile response was observed (fig. 1) that was rapid in onset and transient (the phasic response). After repeated additions of PE in calcium - free buffer the ability to produce even this transient response was lost. Incubation of the tissue in calcium -

FIGURE 3: A characteristic kinetic profile of the contractile response of a desheathed rabbit aorta to different concentrations of PE. PE was added to the organ bath and the increase in tension vs. time was recorded. The drug was washed out after a steady state contraction was obtained and the tissue was allowed to relax (20 - 40 min) to basal tension before the next addition of the drug. Points shown are digitized values traced from the original recording (see under "Methods"). A log concentration - response curve is depicted (inset) for the steady state values observed at each concentration of PE; EC_{50} = 0.2 uM, slope index = 1.16.

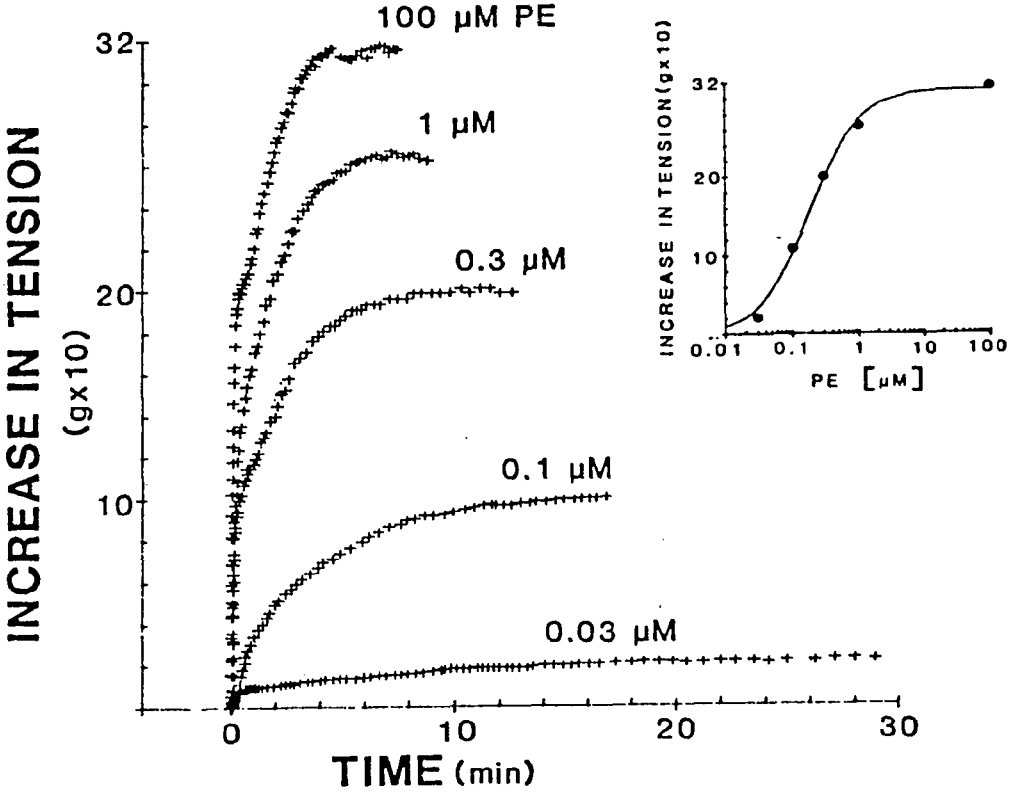
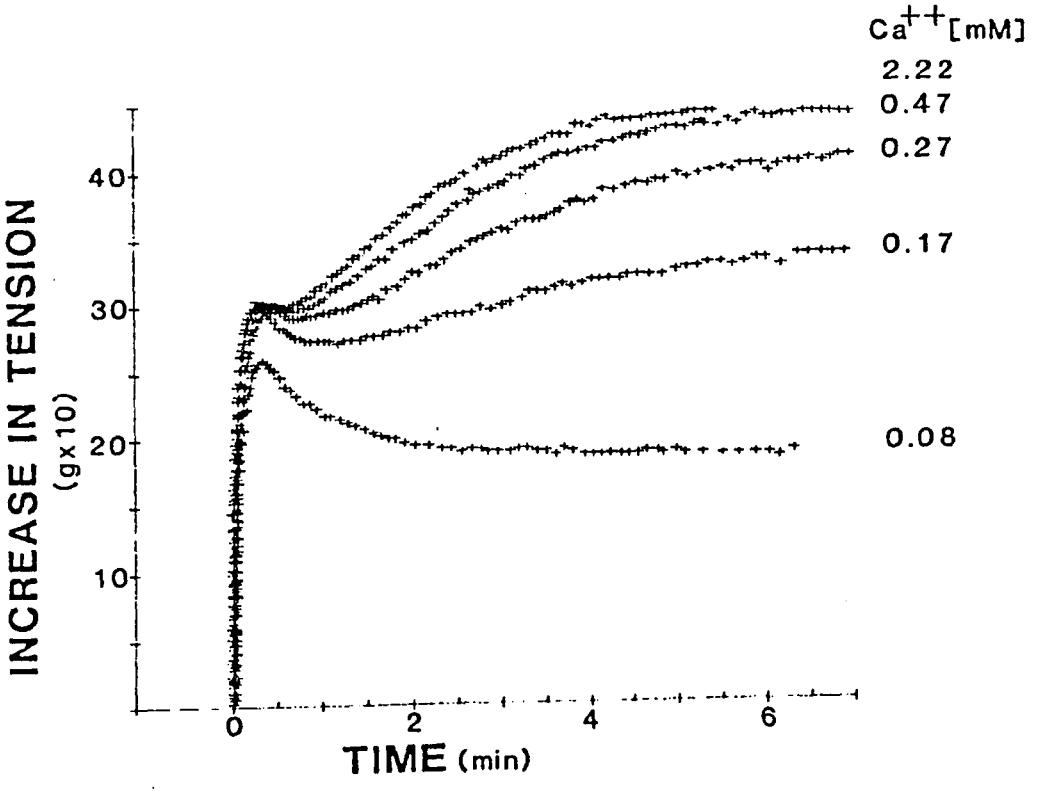

100 μM PE
1 μM
0.3 μM
0.1 μM
0.03 μM
INCREASE IN TENSION
(gx10)
32
20
10
0
10
20
30
TIME (min)
INCREASE IN TENSION(gx10)
32
20
10
0.01
0.1
1
10
100
PE [μM]

FIGURE 4: A characteristic effect of changing buffer [Ca++] on the rabbit aorta contractile response. Two to three minutes before additon of 100 uM PE the buffer calcium concentration was changed to the indicated values. After completion of the agonist - induced response the tissues were again placed in normal Krebs - bicarbonate buffer ([Ca++] = 2.22 mM) for at least 30 min before the next elicited contraction.


Ca++[mM]
2.22
0.47
0.27
0.17
0.08
INCREASE IN TENSION
(g x 10)
40
30
20
10
0
2
4
6
TIME (min)

free buffer for 40 min or longer, without PE additions, also caused the loss of the ability to produce a phasic response. The effects observed in calcium - free buffer were fully reversible , i.e., adding calcium back to the medium always restored the original responsiveness of the tissue, evidenced by identical values of concentration - response curves to that of control.

The phasic response was characterized by manipulating two factors that affect its kinetics: the concentration of agonist and the concentration of calcium in the preincubation buffer.

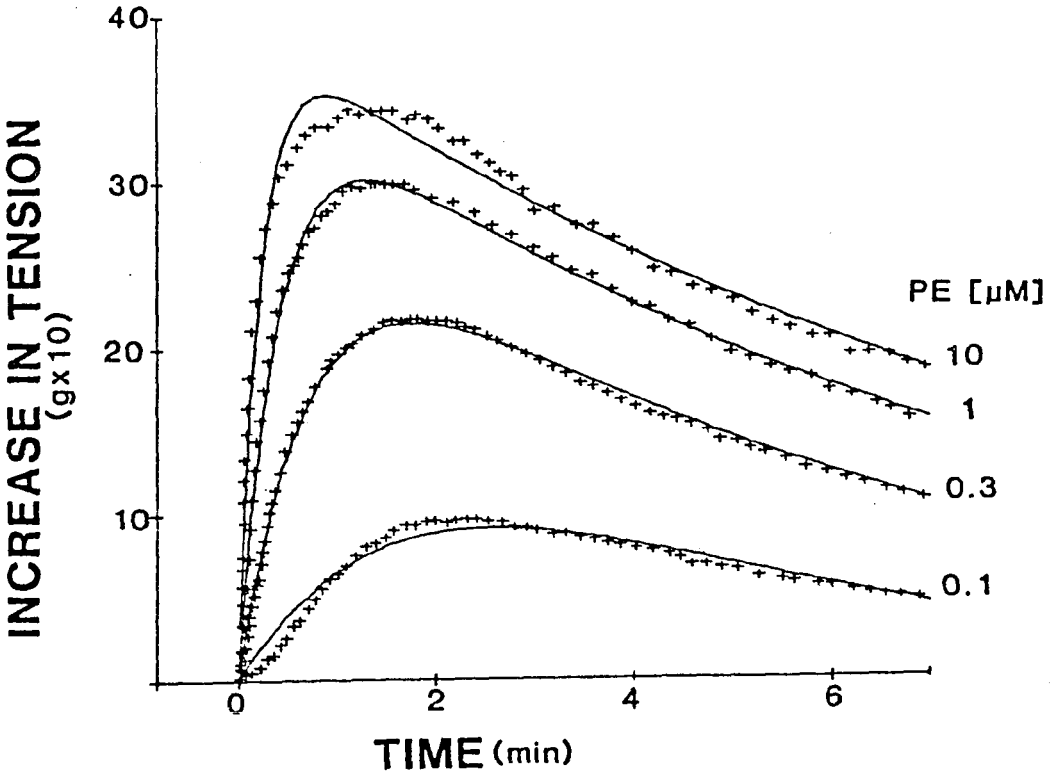
Kinetics of the Phasic Response - Dependence on [PE]:

The observed time course of the contractile response of one ring to a range of PE concentrations (0.1 - 10 uM) is shown in figure 5. The curves in figure 5 are characteristic of a process arising from two sequential steps identifiable as the onset and the subsequent decay of the response. According to this model the behavior should be described by the equation:

$$(21) \qquad R = [(R_0 \cdot k_{on})/(k_{on} - k_{decay})] \cdot [\exp(-k_{decay} \cdot t) - \exp(-k_{on} \cdot t)]$$

where R is the measured response and k_{on} and k_{decay} are the apparent rate constants for the onset and decay of the re-

FIGURE 5: The effect of changing [PE] on the phasic response of the rabbit aorta. The phasic response was generated as described under "Methods" and exemplified in figure 1. The digitized data points of the contractile curves were fitted to equation 21 for the estimation of the rate constants of onset and decay (see data in table 1).


INCREASE IN TENSION
(g x 10)
40
30
20
10
0
2
4
6
TIME (min)
PE [μM]
10
1
0.3
0.1

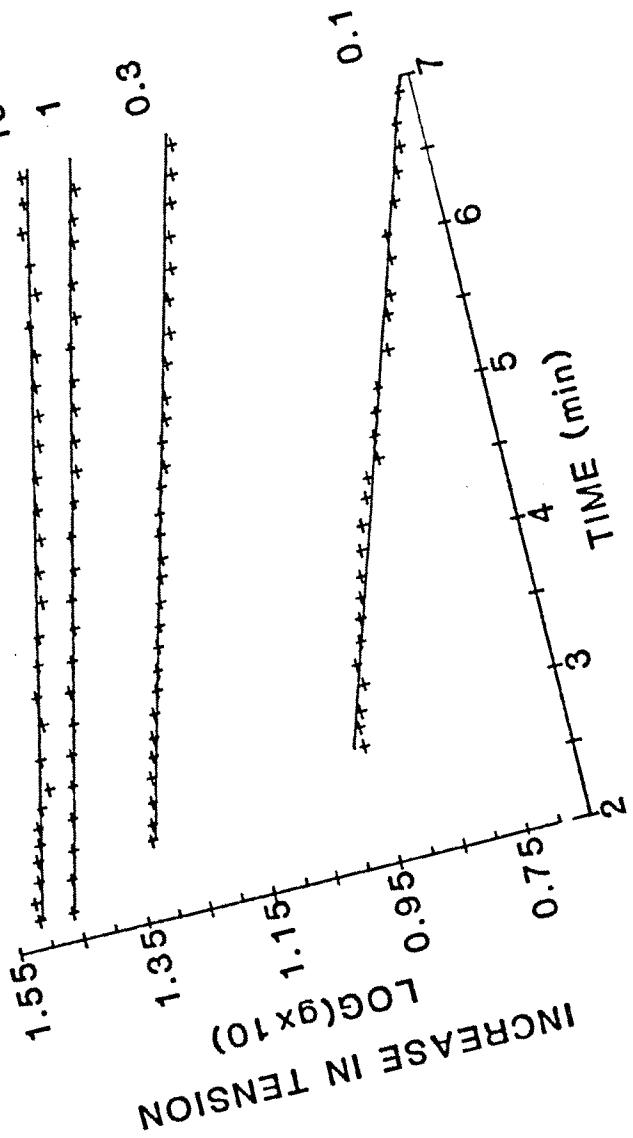
sponse, respectively, and R_0 is the response obtainable under conditions of no decay. This equation was used because it accounts for the major characteristics of the response, i.e., the onset and decay components, and it is not intended to explain all the complexities of the response. Deviations of the fitted curves from the data (e.g., figs. 5 and 7) are the consequence of the approximate nature of this equation.

The data presented in figure 5 reveal that the onset and decay phases are rather well separated in time; the onset usually peaks within 1 to 2 min, while the maximal signal diminshes to half its value in approximately 7 min for all concentrations tested. After the first 3 min the decay process shows first order kinetics independent of the concentration of PE (fig. 6). The decay rate constant can therefore be estimated from a plot of log (R) vs. time, for $t > t_{max}$. The average value of k_{decay} from four concentrations of PE in figure 6 is 0.14 ± 0.01 min^{-1}. In two other experiments k_{decay} was independent of [PE] with average values of k_{decay} equal to 0.12 ± 0.01 min^{-1} (six concentrations) and 0.15 ± 0.02 min^{-1} (six concentrations).

The t_{max} can be obtained from equation 21 by setting the derivative dR/dt to zero and solving for t:

$$(22) \quad t_{max} = 1 / (k_{on} - k_{decay}) \cdot \ln (k_{on} / k_{decay})$$

FIGURE 6: A semilogarithmic transformation of the decay phase (note that the time axis starts at 2 min) of each response in figure 5 was fitted to a straight line. The fitted lines ($r^2 \geq 0.94$) have similar slopes. The average slope is 0.14 ± 0.01 min^{-1}.


PE [μM]
10
1
0.3
0.1
7
6
5
4
3
2
TIME (min)
1.55
1.35
1.15
0.95
0.75
LOG(g x 10)
INCREASE IN TENSION

Because the dependence of t_{max} on the inverse of k_{on} is greater than on ln (k_{on}), it is evident from equation 22 that for a constant k_{decay}, there is an inverse relationship between t_{max} and k_{on}. This can be observed in figure 5 in which the t_{max} decreases as the concentration of PE increases.

Because k_{on} cannot be estimated from a plot of log (R) vs. time, phasic response data for different concentrations of PE were fitted to equation 21. The cumulative results from the fits to five experiments are shown in table 1. The data in table 1 indicate that R_0 is saturable with respect to PE concentration. This behavior is reproducible and gives an average EC_{50} value of 0.17 $\pm$ 0.02 uM and a slope index of 1.11 $\pm$ 0.15 (n = 5). The dependence of k_{on} on PE concentration does not show the same behavior. When the values of k_{on} from three experiments were fitted to a model predicting saturable behavior with respect to [PE] (see equation 17) the values for the EC_{50} converged to 20 uM and 1730 uM in two experiments and did not converge even at a value of 75 mM for the third experiment; the concentration - rate curves had very shallow slopes with slope indices of 0.25, 0.34 and 0.27 respectively. This indicates that k_{on} is not saturable up to a PE concentration of 30 uM.

TABLE 1

Effect of [PE] on the phasic response

Kinetic parameters were estimated by fitting the phasic response curves to equation 21. For all fits $r^2 \geq 0.97$. An analysis of variance of the fitted data indicates that for all fits the F ratio was significant at the 0.001 level.

[PE]	$R_0/R_0max \pm$ S.E.M.[a]	$k_{on} \pm$ S.E.M.	$k_{decay} \pm$ S.E.M.[b]	(n)
uM			min^{-1}	
0.03	0.12 ± 0.01	1.5 ± 0.4	0.18 ± 0.05	(4)
0.1	0.40 ± 0.03	1.7 ± 0.4	0.18 ± 0.03	(5)
0.3	0.65 ± 0.06	2.5 ± 0.6	0.18 ± 0.01	(5)
1.0	0.87 ± 0.06	3.4 ± 0.6	0.14 ± 0.01	(5)
3.0	0.95 ± 0.03	4.5 ± 0.7	0.18 ± 0.04	(5)
10.0	0.96 ± 0.01	5.9 ± 1.1	0.10 ± 0.01	(5)
30.0	0.99 ± 0.03	10.5 ± 2.6	0.15 ± 0.02	(2)

[a] The mean response from (n) experiments at each concentration of PE expressed as a fraction of the maximal response (R_0max) to PE. The EC_{50} determined from five experiments, is 0.17 ± 0.02 uM, the average slope index of the concentration response curves is 1.11 ± 0.15.

Kinetics of the Phasic Response - Dependence [Ca++]:

A second series of experiments was conducted to characterize the dependence of the phasic response on the preincubation concentration of calcium. A preincubation period of 30 to 40 min was sufficient to produce a consistent and reproducible difference in the kinetics of the PE - induced contraction. Representative results are shown in figure 7. The curves in figure 7 are similar in shape to those of figure 5. The average value of k_{decay} from the plot log (R) vs. time, for eight concentrations of calcium, was 0.15 $\pm$ 0.02 min^{-1}; similar values (0.15 $\pm$ 0.01 min^{-1} for seven concentrations and 0.14 $\pm$ 0.02 min^{-1} for four concentrations) were obtained from two other experiments. No dependence of k_{decay} on the concentration of calcium was observed. The digitized data were fitted to equation 21. R_0 shows a saturable dependence on the concentration of calcium (table 2). This behavior is reproducible and gives an average EC_{50} value of 0.09 $\pm$ 0.02 mM and a slope index of 0.98 $\pm$ 0.25 (n = 4). The apparent rate constant k_{on} has an average value of 3.2 $\pm$ 0.1 min^{-1} for the data shown in table 2 and there appears to be no dependence of k_{on} on [Ca++].

FIGURE 7: The effect of changing preincubation [Ca++] on the phasic response to 1 uM PE. The response was generated as described under "Methods" and exemplified in figure 1. The digitized data points of the contractile curves were fitted to equation 21 for the estimation of the rate constants of onset and decay (see data in table 2).

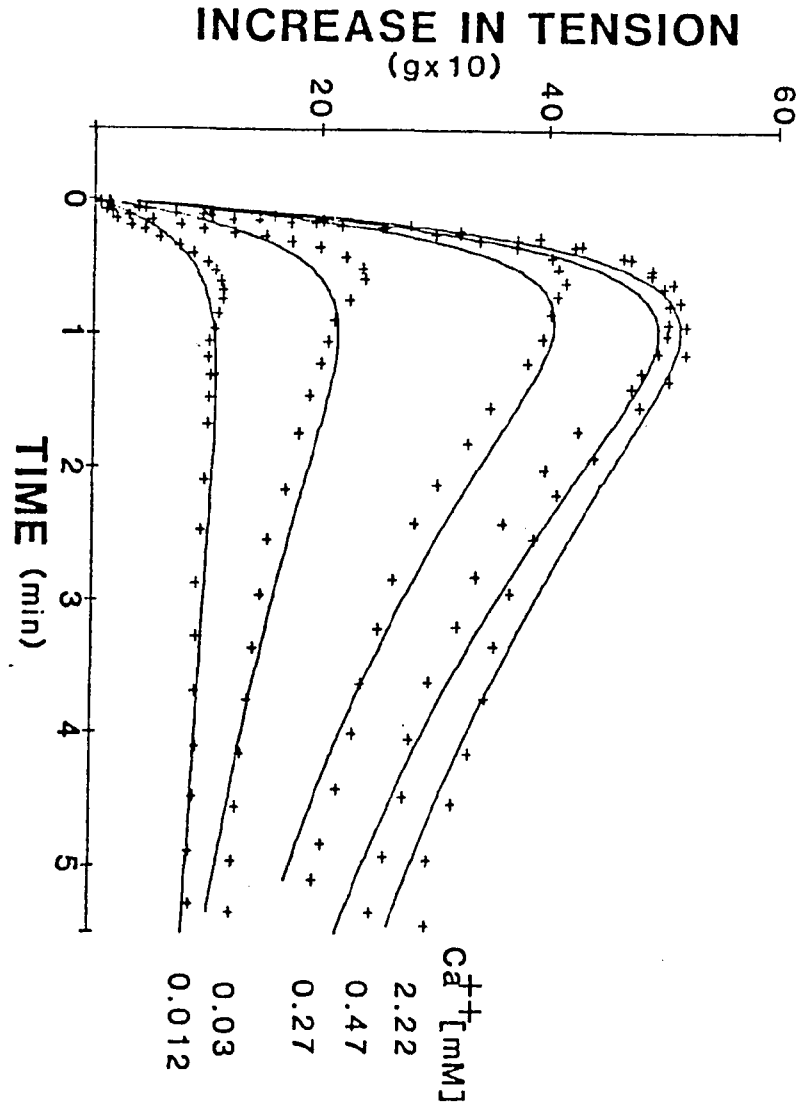

INCREASE IN TENSION
(gx10)
20
40
60
0
1
2
3
4
5
TIME (min)
Ca++[mM]
2.22
0.47
0.27
0.03
0.012

TABLE 2

Effect of preincubation [Ca++] on the phasic response to 1 uM PE

Kinetic parameters were estimated by fitting the phasic response curves to equation 21. For all fits $r^2 \geq 0.97$. An analysis of variance of the fitted data indicates that for all fits the F ratio was significant at the 0.001 level.

[Ca++]	$R_0/R_0max \pm$ S.E.M. [a]	$k_{on} \pm$ S.E.M. [b]	$k_{decay} \pm$ S.E.M. [c]	(n)
mM		min^{-1}		
0.012	0.27 ± 0.08	3.0 ± 0.1	0.14 ± 0.08	(2)
0.03	0.31 ± 0.06	3.6 ± 0.3	0.17 ± 0.02	(3)
0.08	0.46 ± 0.10	3.3 ± 0.5	0.21 ± 0.03	(4)
0.27	0.69 ± 0.08	3.0 ± 0.5	0.23 ± 0.03	(3)
0.47	0.87 ± 0.03	3.2 ± 0.9	0.23 ± 0.04	(3)
2.22	0.91 ± 0.02	2.9 ± 0.2	0.16 ± 0.02	(4)

[a] The mean response from (n) experiments at each concentration of calcium expressed as a fraction of the maximal response (R_0max) to calcium. The EC_{50} determined from four experiments, is 0.09 ± 0.02 mM, the average slope index of the concentration response curves is 0.98 ± 0.25.

[b] The average k_{on} for the values in table 2 is 3.2 ± 0.1 min^{-1}.

[c] The average k_{decay} for the values in table 2 is 0.19 ± 0.02 min^{-1}.

Kinetics of the Tonic Response - Dependence on [PE]:

By exhausting the ability of the tissue to produce a phasic response in calcium - free buffer a monophasic tonic response could be elicited with the addition of PE followed by calcium (fig. 2). Figure 8 shows the dependence of the tonic response on the concentration of PE. If the interval between the agonist and calcium additions was lengthened from 10 sec to 2 min, employing the same concentration of agonist and calcium, there was no observable change in the onset rate or magnitude of the tonic response. Both the amplitude of the response (R_{eq}) and the rate constant of onset (k_{obs}) depend on PE concentration. A plot of the fractional response, ($R_{eq}/R_{eq}max$), vs. [PE] (fig. 9), shows a saturable curve. This behavior is reproducible and gives an average EC_{50} of 0.13 $\pm$ 0.07 uM and a slope index of 1.34 $\pm$ 0.11 (n = 6). The EC_{50} did not differ significantly in an experiment where the concentration of calcium in the buffer was changed from 2.22 mM (EC_{50} = 0.09 mM) to 0.47 mM (EC_{50} = 0.05 mM) or to 0.08 mM (EC_{50} = 0.10 mM).

To analyze the dependence of the rate of onset on [PE], first the nature of the kinetics of the tonic response was determined. At very short times the onset of the tonic response tends to be sigmoidal (e.g., fig. 8, curve for 0.06 uM PE) but the rest of the curve is compatible with first order kinetics and fits the function (fig. 10),

FIGURE 8: The effect of changing [PE] on the tonic response of the rabbit aorta. The response was generated as described under "Methods" and exemplified in figure 2.

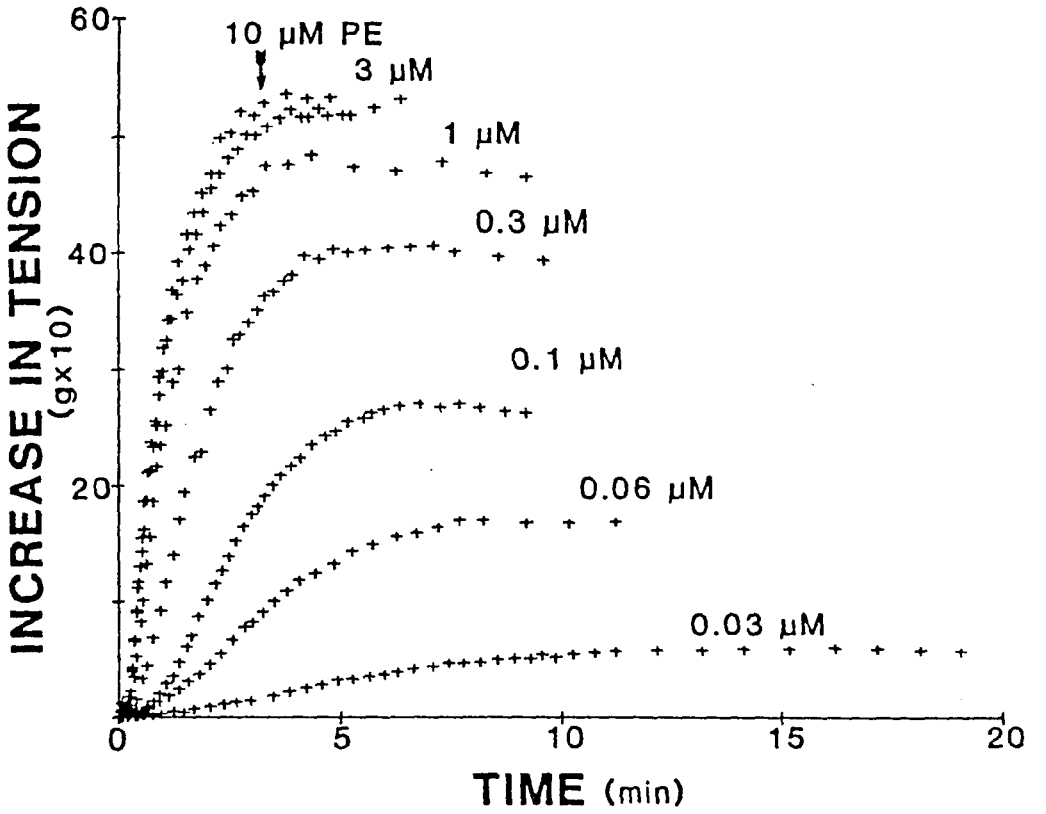

60
40
20
0
5
10
15
20
10 μM PE
3 μM
1 μM
0.3 μM
0.1 μM
0.06 μM
0.03 μM
INCREASE IN TENSION (gx10)
TIME (min)

FIGURE 9: The concentration - response curve for the equilibrium response (R_{eq}) values of the tonic response expressed as a fraction of the maximal response to PE ($R_{eq}/R_{eq}max$). The curve is constructed using the geometric mean of the EC_{50} values (0.13 $\pm$ 0.07 uM) and the arithmetic mean of the slope indices (1.34 $\pm$ 0.11) from six experiments. The open circles are the mean fractional responses $\pm$ S.E.M. of the observed data from (n) replicate experiments.

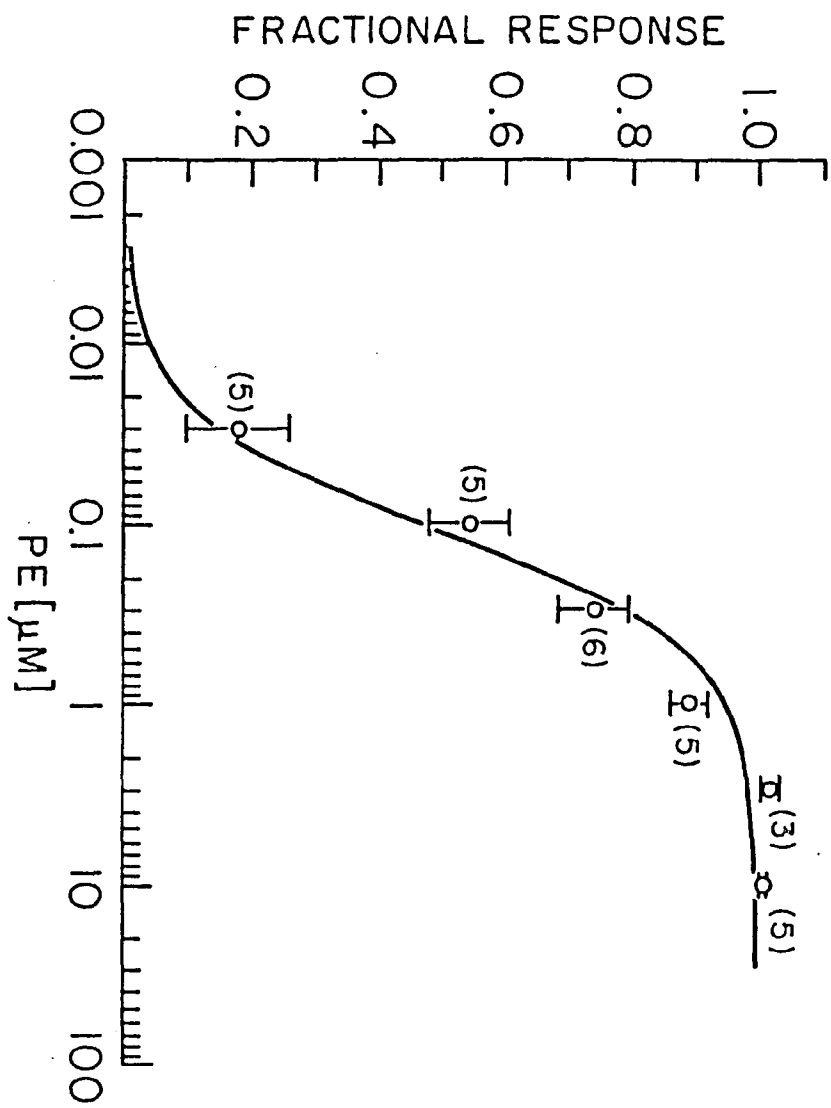
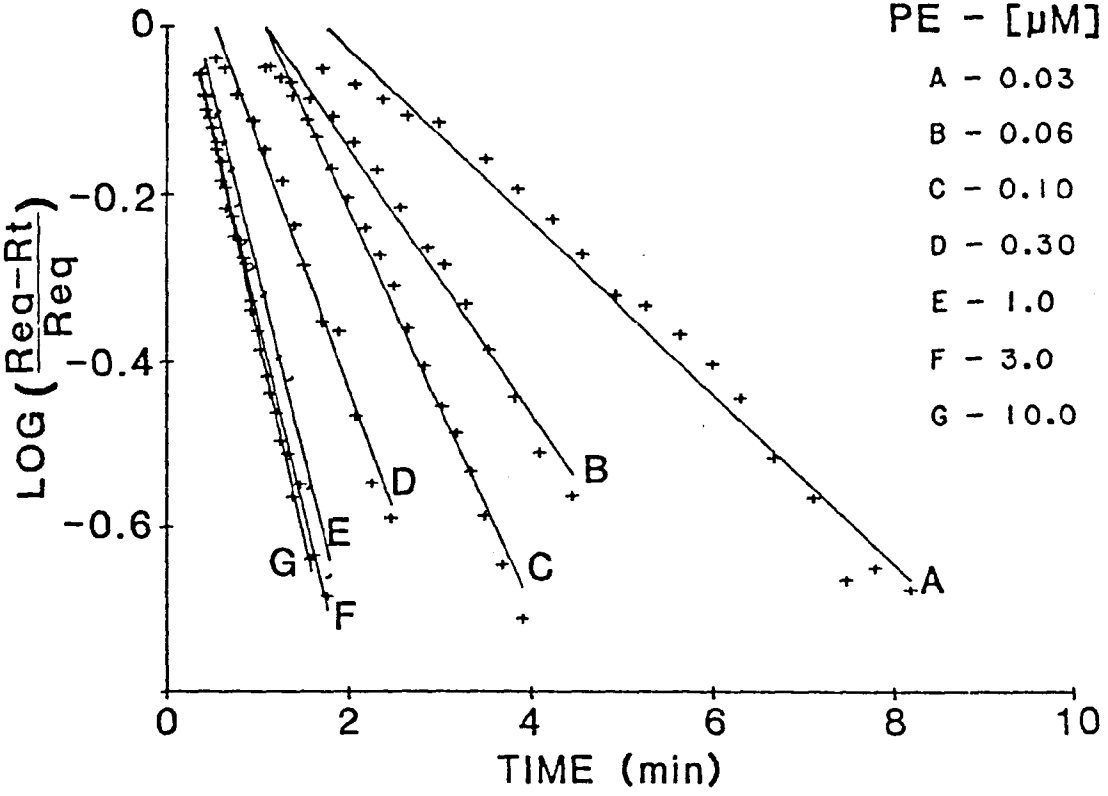

FRACTIONAL RESPONSE
0.2
0.4
0.6
0.8
1.0
0.001
0.01
0.1
1
10
100
PE [μM]
(5)
(5)
(6)
(5)
(3)
(5)

FIGURE 10: A semilogarithmic transformation of the onset of each response in figure 8 fitted to straight line. The slopes of the fitted lines, which provide estimates of the rate constants at each concentration of PE, are from regression analyses all having $r^2 \geq 0.98$.


PE - [μM]
A - 0.03
B - 0.06
C - 0.10
D - 0.30
E - 1.0
F - 3.0
G - 10.0
LOG (Req-Rt / Req)
0
-0.2
-0.4
-0.6
0
2
4
6
8
10
TIME (min)
A
B
C
D
E
F
G

(23) $$\ln [(R_{eq} - R_t)/R_{eq}] = -k_{obs} \cdot t$$

where R_{eq} and R_t are the levels of response at equilibrium and at time t, respectively, and k_{obs} is the rate constant of onset. It appears that k_{obs} reaches a limiting value ($k_{obs}max$) at increasing concentrations of PE and a plot of fractional k_{obs}, ($k_{obs}/k_{obs}max$), vs. log [PE] (fig. 11), indicates that k_{obs} depends on the concentration of PE in a saturable manner. The EC_{50} for k_{obs} vs. [PE] of 0.18 $\pm$ 0.12 uM (n = 6) is similar to the EC_{50} for R_{eq} vs. [PE]. Because of its saturable dependence on [PE], k_{obs} can be considered as a pseudo - first order rate constant with respect to DR:

(24) $$k_{obs} = k_{int} \cdot [DR]$$

where k_{int} is the intrinsic second order rate constant. If DR is expressed (Clark, 1933) as,

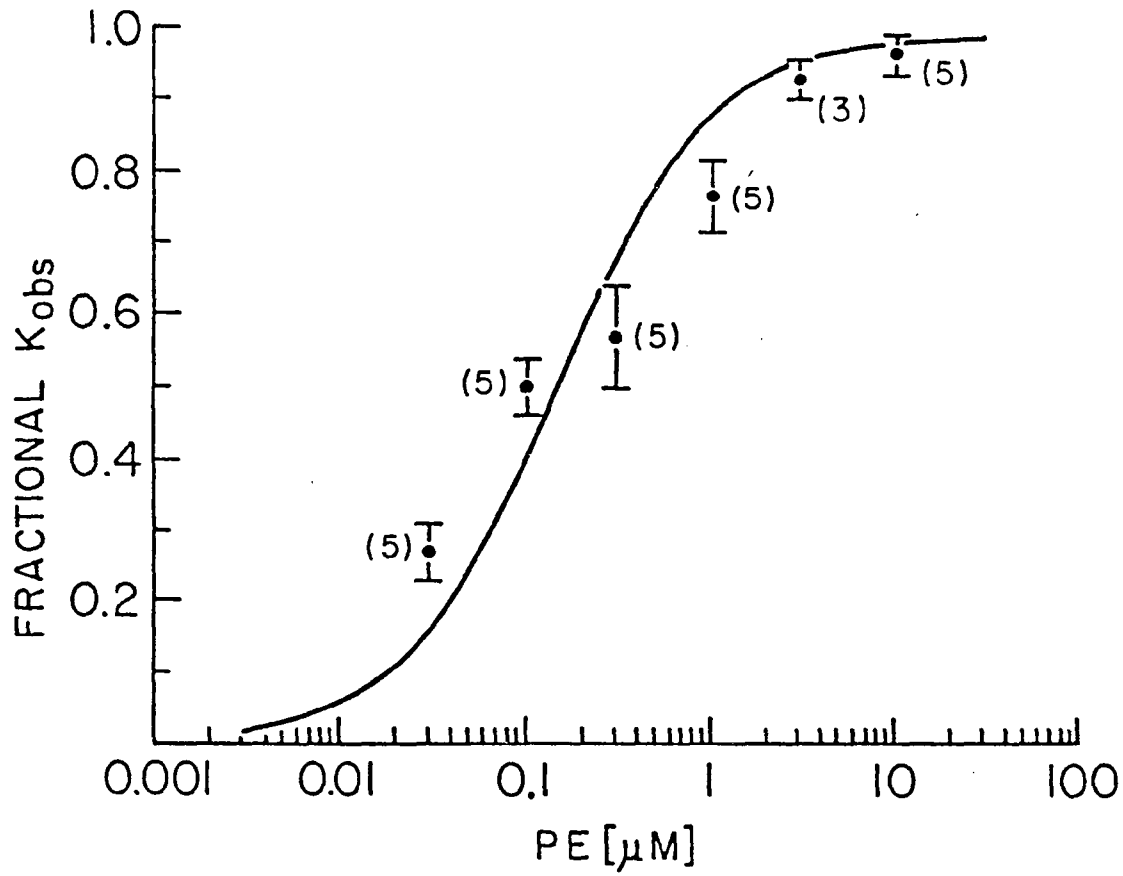
(25) $$[DR] = (R_{tot} [D])/([D] + K_D)$$

where R_{tot} is the maximal number of receptors for the drug, D, and K_D is the dissociation constant, then substituting equation 24 into equation 25 gives a saturable dependence of k_{obs} on the concentration of drug:

(26) $$k_{obs} = k_{obs}max\ [D]/([D] + K_D)$$

where K_D is the EC_{50} of the concentration - rate curve (k_{obs} vs. [D]) and $k_{obs}max = k_{int} \cdot R_{tot}$.

FIGURE 11: The dependence of the fractional rate constant ($k_{obs}/k_{obs}max$) of the tonic response on [PE]. The curve is constructed using the geometric mean of the EC_{50} values (0.18 $\pm$ 0.12 uM) and the arithmetic mean of the slope indices (1.05 $\pm$ 0.24) from six experiments. The closed circles are the mean fractional response $\pm$ S.E.M. of the observed data from (n) replicate experiments.


1.0
0.8
0.6
0.4
0.2
FRACTIONAL K_{obs}
(5)
(5)
(5)
(5)
(3)
(5)
0.001
0.01
0.1
1
10
100
PE [μM]

The results from four different measurements of $k_{obs}max$ are 1.36, 1.30, 1.37, 1.22 min^{-1}. Two other experiments gave substantially lower (by 30 - 40%) values for $k_{obs}max$. These lower values for $k_{obs}max$ may reflect a lower concentration of receptors in these two preparations.

Kinetics of the Tonic Response - Dependence on [Ca++]:

The dependence of the kinetics of the tonic response at 1 uM PE and its equilibrium level on the concentration of extracellular calcium is shown in figure 12. The level of response saturates with increasing levels of extracellular calcium. The estimated EC_{50} value of the response level as a function of [Ca++] (fig. 13) is 0.20 $\pm$ 0.02 mM (n = 4) and is independent of [PE]. The analysis of the kinetics of response generation (figs. 14 and 15) shows that this process is also first order for a constant concentration of calcium. Using equation 23 one obtains a series of pseudo - first order rate constants, k_{obs}, for different values of extracellular [Ca++]. k_{obs} shows a dependence on [Ca++] which may reflect a dependence of the intrinsic rate constant, k_{int} on the concentration of available calcium. Here however the dependence of k_{obs} on the concentration of calcium is nonsaturable and is essentially linear between 0.27 and 2.22 mM calcium (n = 4, fig. 15). At very low con-

FIGURE 12: The effect of changing buffer [Ca++] on the tonic response of the rabbit aorta to 1 uM PE. See under "Methods" for details.

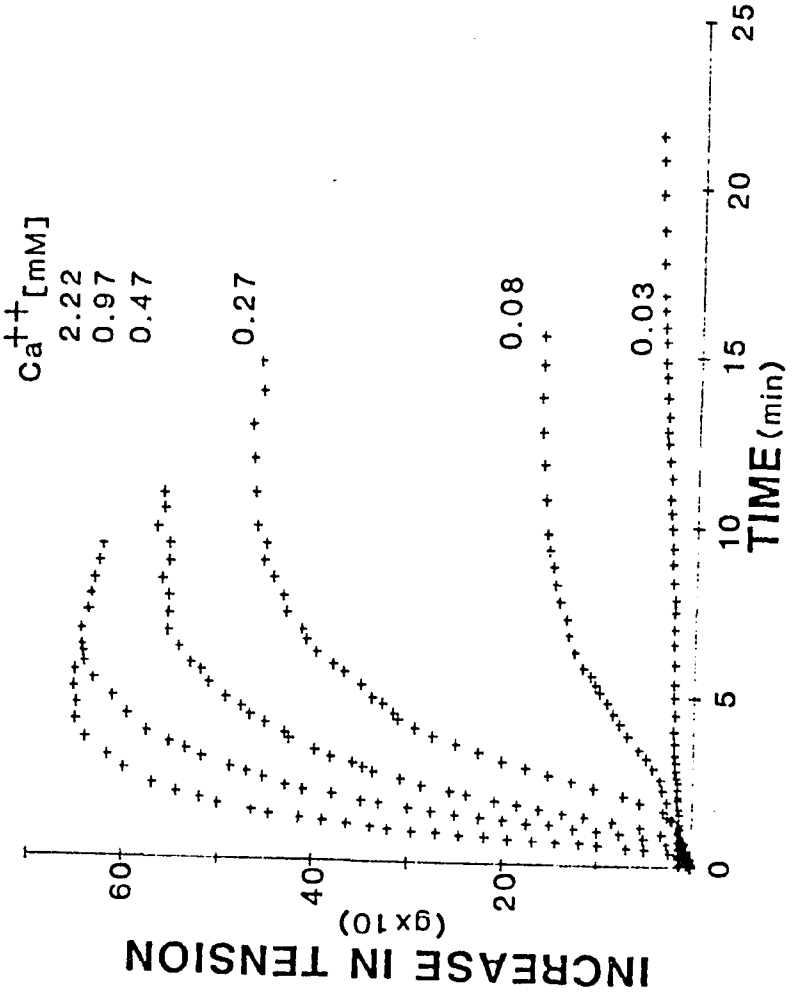

INCREASE IN TENSION
(gx10)
60
40
20
0
5
10
15
20
25
TIME (min)
Ca^{++} [mM]
2.22
0.97
0.47
0.27
0.08
0.03

FIGURE 13: The concentration - response curve for the equilibrium response (R_{eq}) values of the tonic response expressed as a fraction of the maximal response to calcium at [PE] = 1 uM. The curve is constructed using the geometric mean of the EC_{50} values (0.20 $\pm$ 0.02 mM) and the arithmetic mean of the slope indices (1.85 $\pm$ 0.26) from four experiments. The open circles are the mean fractional responses $\pm$ S.E.M. of the observed data from (n) replicate experiments.

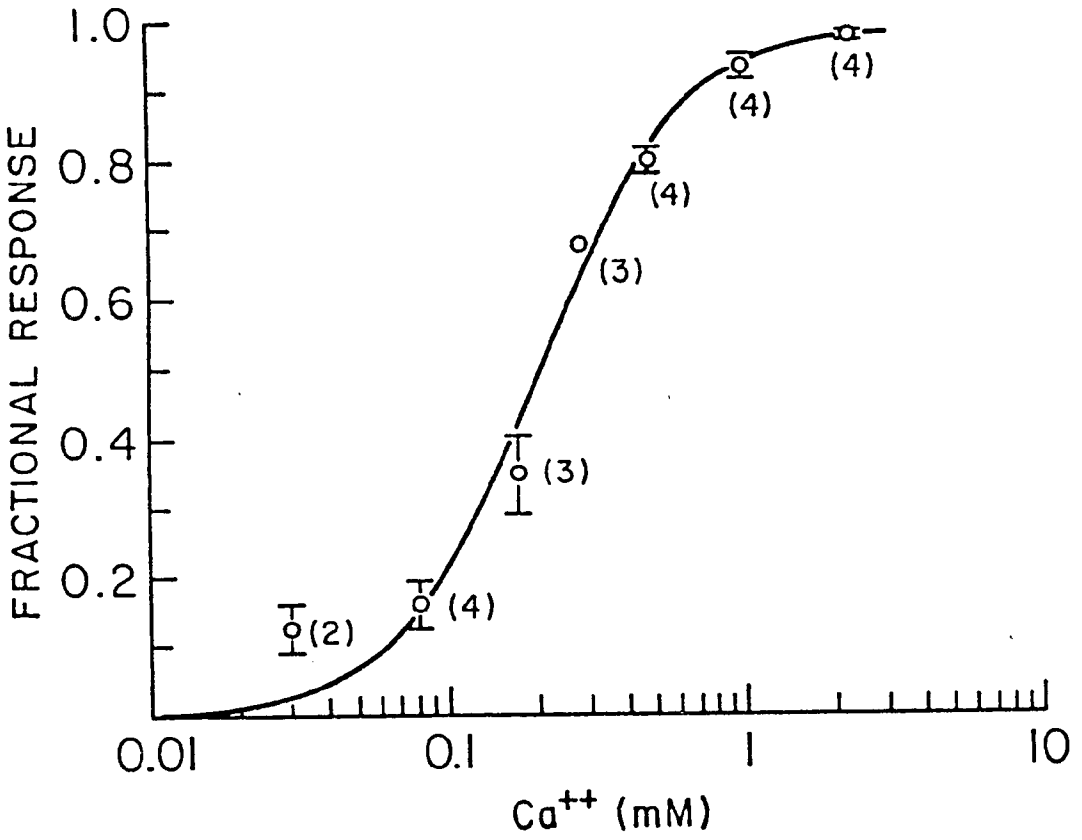

FRACTIONAL RESPONSE
1.0
0.8
0.6
0.4
0.2
(2)
(4)
(3)
(3)
(4)
(4)
(4)
0.01
0.1
1
10
Ca++ (mM)

FIGURE 14: A semilogarithmic transformation of the onset of each response in figure 12 fitted to a straight line. The slopes of the fitted lines, which provide estimates of the rate constants at each concentration of calcium, are from regression analyses all having $r^2 \geq 0.94$.

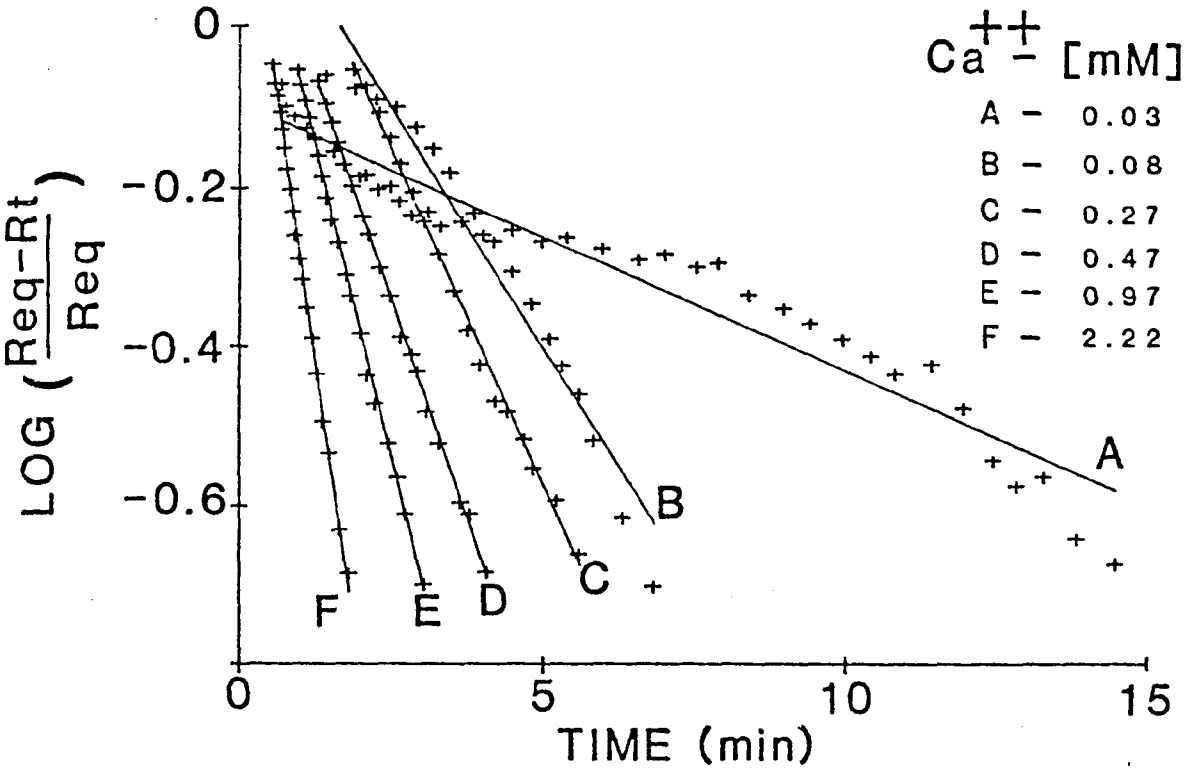
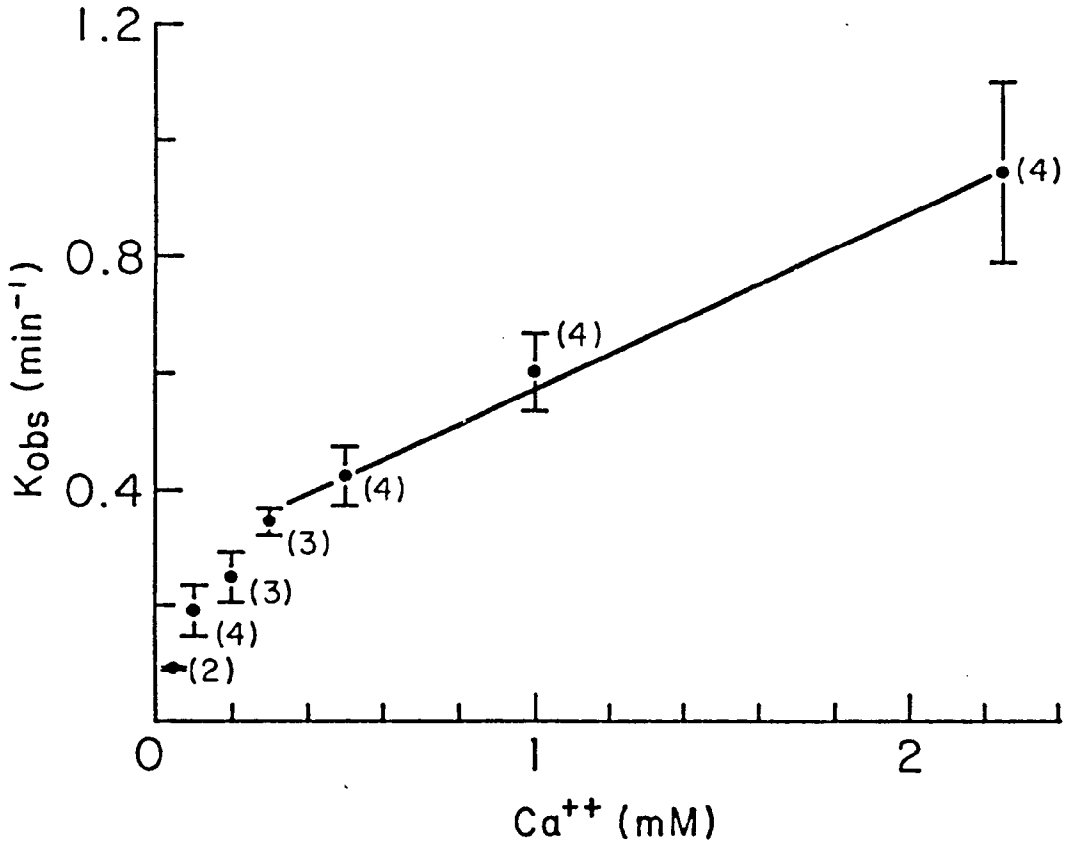

0
-0.2
-0.4
-0.6
LOG (Req-Rt / Req)
0
5
10
15
TIME (min)
Ca++ [mM]
A - 0.03
B - 0.08
C - 0.27
D - 0.47
E - 0.97
F - 2.22
A
B
C
D
E
F

FIGURE 15: A graph depicting the dependence of the k_{obs} of the tonic response at [PE] = 1 uM on [Ca++]. The closed circles are the mean k_{obs} $\pm$ S.E.M. of the observed data, from four experiments, at each concentration of calcium. A line is fitted (r^2 = 0.99) to the points between 0.27 and 2.22 mM calcium to illustrate the linear relationship between k_{obs} and [Ca++] in this range.


1.2
0.8
0.4
0
1
2
K_{obs} (min^{-1})
Ca^{++} (mM)
(4)
(4)
(4)
(3)
(3)
(4)
(2)

centrations of calcium ([Ca++] < 0.27 mM), some deviation from the linear dependence on extracellular calcium is seen.

PART II. KINETIC CHARACTERIZATION OF THE SEROTONERGIC RESPONSE

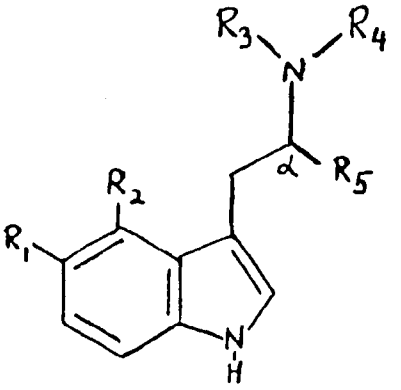
The kinetic analysis of the PE contractile response revealed that the relationship between k_{obs} and the concentration of PE can be described by equation 26. At a saturating concentration of D (when [D] >> K_D) k_{obs} becomes equal to $k_{obs}max$. As shown in equation 26 the maximal observed rate constant ($k_{obs}max$) should depend on both the intrinsic rate constant (k_{int}) and the total number of receptors (R_{tot}). Thus, $k_{obs}max$ reflects both a property of the drug and a property of the tissue. The principal objective of the studies described below is to probe the dependence of $k_{obs}max$ on the nature of the drug and on the size of the receptor population.

The following studies were carried out on responses mediated by both adrenergic and serotonergic receptors. The kinetic analysis was applied to the serotonergic system for two reasons: 1) it is important to know if the kinetic behavior of the adrenergic response can be generalized to another receptor in the same tissue and 2) the studies on the dependence of $k_{obs}max$ on k_{int} require that the kinetics of drugs acting at the same receptor but generating different stimuli be compared. The selection of alpha - adrenergic agonists with different stimuli is rather limited; epinephrine, norepinephrine, and PE all have

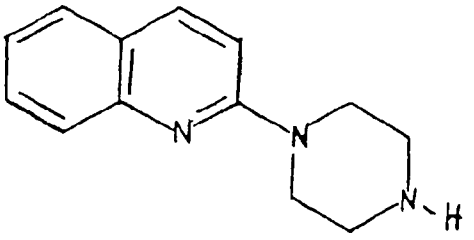
relative efficacies close to 1 (Besse and Furchgott, 1976). There is, on the other hand, a selection of serotonergic agonists that elicit contractions via the 5-HT receptor in the rabbit aorta with varying efficacies (see table 14). The following indole alkylamines, shown in figure 16, were tested: 5-hydroxytryptamine (5-HT), tryptamine (Tryp), alpha - methyltryptamine (AMT), N - methyltryptamine (NMT), and dimethyltryptamine (DMT). Clancy et al. (1983, 1984) demonstrated the 5-HT receptor specificity of NMT, AMT and DMT. Quipazine was also tested as a drug that elicits a contractile respone through the 5-HT receptor (Stollak, 1982) and does not contain the indole nucleus. Although tryptamine is 5-HT receptor selective it may also elicit a weak contraction via the adrenergic receptor (Stollak and Furchgott, 1983). This adrenergic component of tryptamine and possibly of the other tryptamine analogues was eliminated by pretreating the tissue with benetrexamine HCl to inactivate the adrenergic receptors (Stollak and Furchgott, 1983).

The kinetic profiles of the contractile responses of a rabbit aorta ring to a saturating concentration (15 uM) of 5-HT, AMT, and NMT are shown in figure 17. Similar to the response elicited by PE (see fig. 3), the response to each of these drugs is biphasic and is composed of an initial phasic component followed by a tonic component. The kinetic

FIGURE 16: The structures of the six serotonergic agonists used in these studies: 5-hydroxytryptamine (5-HT), tryptamine (Tryp), alpha - methyltryptamine (AMT or ∝MT), N - methyltryptamine (NMT), dimethyltryptamine (DMT), and quipazine (Quip).

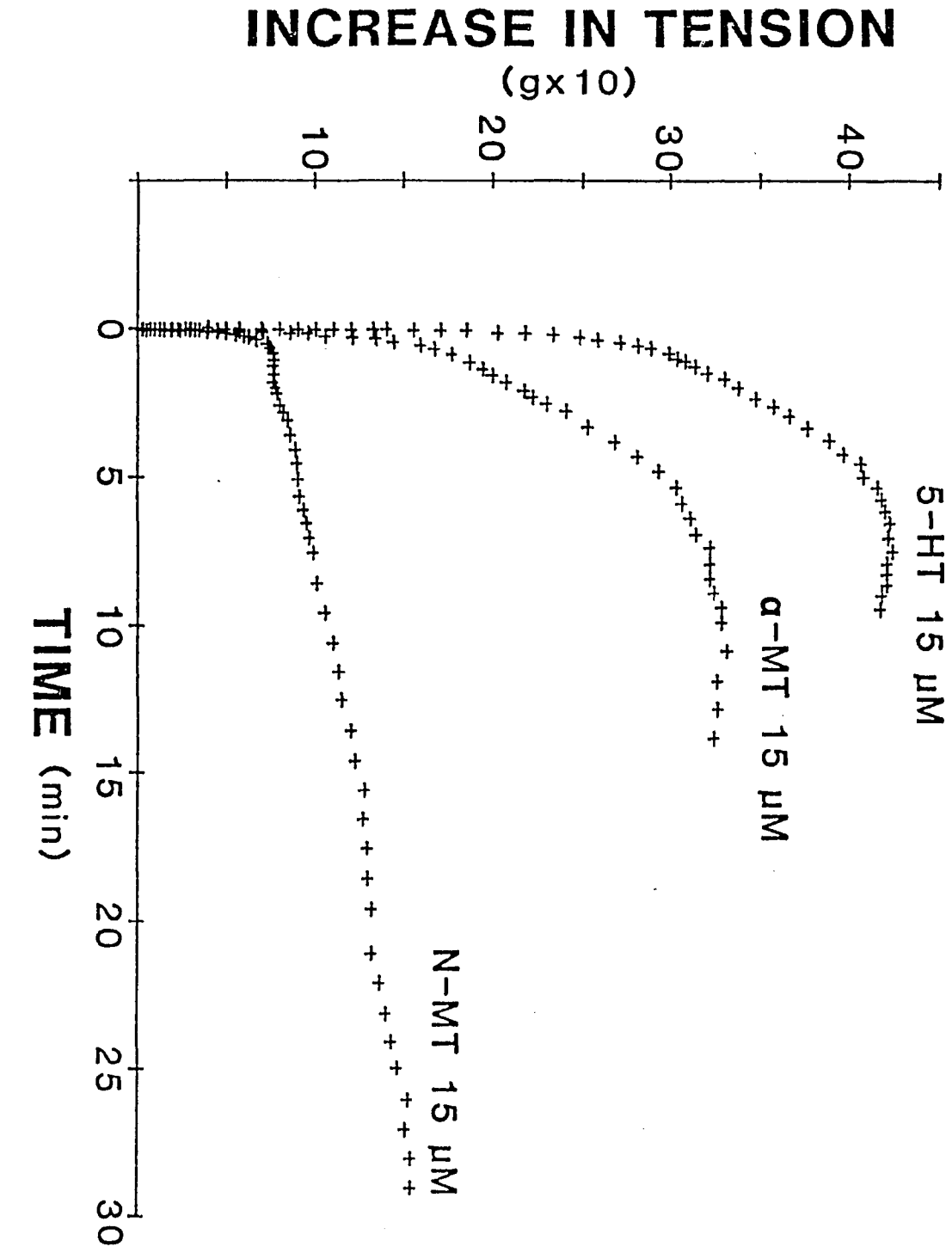


Drug	R_1	R_2	R_3	R_4	R_5
5-HT	OH	H	H	H	H
Tryp	H	H	H	H	H
αMT	H	H	H	H	CH_3
NMT	H	H	H	CH_3	H
DMT	H	H	CH_3	CH_3	H



Quipazine : [2-(1-piperazinyl) quinoline]

FIGURE 17: A characteristic kinetic profile of the contractile responses of a desheathed rabbit aorta to a saturating concentration (15 uM) of three serotonergic agonists: 5-hydroxytryptamine (5-HT), alpha - methyltryptamine (AMT), and N - methyltryptamine (NMT).


INCREASE IN TENSION
(gx10)
10
20
30
40
0
5
10
15
20
25
30
TIME (min)
5-HT 15 μM
α-MT 15 μM
N-MT 15 μM

characterization of the phasic and tonic responses to two of these drugs, 5-HT and NMT, is described below.

Characterization of the Phasic Response to 5-HT and NMT:

The phasic response to 5-HT and NMT can be elicited using the same procedure used to generate the phasic response to PE. The effect of changing the concentration of agonist on the phasic response to 5-HT and NMT was investigated. Tables 3 and 4 show the cumulative data for the phasic responses fitted to equation 21. The values of R_0 saturate with increasing agonist concentration for both 5-HT and NMT. At saturating concentrations (> 50 times the EC_{50}) a progressive diminution of the response level can be observed. This may be attributed to a concentration dependent relaxation of the 5-HT contractile response (Maayani and Osman, private communication). The average EC_{50} of NMT is 6.13 $\pm$ 1.07 uM (n = 5) and of 5-HT is 0.22 $\pm$ 0.03 uM (n = 5). Similar to the observations for PE, the rate constant of onset, k_{on}, for 5-HT or NMT does not appear to saturate at a concentration of agonist 50 times that of the EC_{50} (when the k_{on} values from some experiments were fitted to an equation predicting saturability with respect to [NMT] or [5-HT] (see equation 17) the EC_{50} value for k_{on} vs. [NMT] did not converge at 200 mM; the EC_{50} values for k_{on} vs. [5-HT] converged at 3.7 uM and 1139 uM; the concen-

TABLE 3

Effect of [5-HT] on the phasic response

Kinetic parameters were estimated by fitting the phasic response curves to equation 21. For all fits $r^2 \geq 0.97$. An analysis of variance of the fitted data indicates that for all fits the F ratio was significant at the 0.001 level.

[5-HT]	$R_0/R_0max \pm$ S.E.M.[a]	$k_{on} \pm$ S.E.M.	$k_{decay} \pm$ S.E.M.[b]	(n)
uM			min^{-1}	
0.03	0.11	0.86	0.11	(1)
0.1	0.27 ± 0.08	1.4 ± 0.2	0.12 ± 0.07	(3)
0.3	0.61 ± 0.07	1.8 ± 0.2	0.17 ± 0.07	(5)
1.0	0.77 ± 0.09	2.3 ± 0.3	0.20 ± 0.04	(5)
3.0	0.89 ± 0.06	3.0 ± 0.2	0.15 ± 0.01	(4)
10.0	0.90 ± 0.04	3.4 ± 0.2	0.18 ± 0.03	(3)
30.0	0.70 ± 0.12	3.7 ± 0.1	0.26 ± 0.05	(2)
100.0	0.66 ± 0.16	5.1 ± 0.3	0.27 ± 0.01	(2)

[a] The mean response from (n) experiments at each concentration of 5-HT expressed as a fraction of the maximal response (R_0max) to 5-HT. The EC_{50} determined from five experiments, is 0.22 ± 0.03 uM, the average slope index of the concentration response curves is 1.37 ± 0.14.

[b] The average k_{decay} for the values in table 3 is 0.18 ± 0.02 min^{-1}.

TABLE 4

Effect of [NMT] on the phasic response

Kinetic parameters were estimated by fitting the phasic response curves to equation 21. For all fits $r^2 \geq 0.97$. An analysis of variance of the fitted data indicates that for all fits the F ratio was significant at the 0.001 level.

[NMT]	$R_0/R_0max \pm$ S.E.M.[a]	$k_{on} \pm$ S.E.M.	$k_{decay} \pm$ S.E.M.[b]	(n)
uM		min^{-1}		
1.0	0.16 ± 0.04	0.9 ± 0.2	0.13 ± 0.03	(3)
3.0	0.34 ± 0.04	1.7 ± 0.3	0.17 ± 0.03	(5)
10.0	0.63 ± 0.05	2.1 ± 0.4	0.25 ± 0.08	(5)
30.0	0.94 ± 0.09	3.2 ± 0.4	0.44 ± 0.15	(3)
100.0	1.05 ± 0.02	3.9 ± 0.5	0.29 ± 0.02	(2)
300.0	0.91 ± 0.04	8.0 ± 3.1	0.24 ± 0.02	(2)

[a] The mean response from (n) experiments at each concentration of NMT expressed as a fraction of the maximal response (R_0max) to NMT. The EC_{50} determined from five experiments, is 6.13 ± 1.07 uM, the average slope index of the concentration response curves is 1.12 ± 0.18.

[b] The average k_{decay} for the values in table 4 is 0.25 ± 0.04 min^{-1}.

tration - rate curves had shallow slopes with slope indices of 0.52, 0.47, and 0.21, respectively. The k_{decay} of either agonist is independent of the agonist concentration. The average k_{decay} of 5-HT is 0.18 $\pm$ 0.02 min^{-1} and of NMT is 0.25 $\pm$ 0.04 min^{-1} and are similar to the values measured for PE.

Characterization of the Tonic Response to 5-HT and NMT:

The tonic response to 5-HT and NMT was elicited using the same procedure utilized to generate the tonic response to PE. Both the amplitude of the response (R_{eq}) and the rate constant of response generation (k_{obs}) depend on the concentration of agonist. A plot of the response R_{eq} vs. [5-HT] (fig. 18) and R_{eq} vs. [NMT] (fig. 19) from individual rings reveal saturable curves. This behavior is reproducible and gives an average EC_{50} for 5-HT of 0.056 $\pm$ 0.011 uM and a slope index of 1.71 $\pm$ 0.22 (n = 4) and an average EC_{50} of 0.79 $\pm$ 0.10 uM and a slope index of 1.45 $\pm$ 0.12 (n = 4) for NMT. These values are less than the respective EC_{50} values of the phasic response parameter R_0 vs. [5-HT] or [NMT] (see table 5).

The kinetic analysis of the curves revealed that the responses approximate first order kinetics and can be fitted to the function expressed in equation 23. A plot of k_{obs} vs. [5-HT] (fig. 20) or vs. [NMT] (fig. 21) shows a satur-

FIGURE 18: A concentration - response curve depicting the dependence of the equilibrium response (R_{eq}) values of the tonic response on [5-HT] in an individual aortic ring. The EC_{50} is 0.074 uM and the slope index is 1.49.

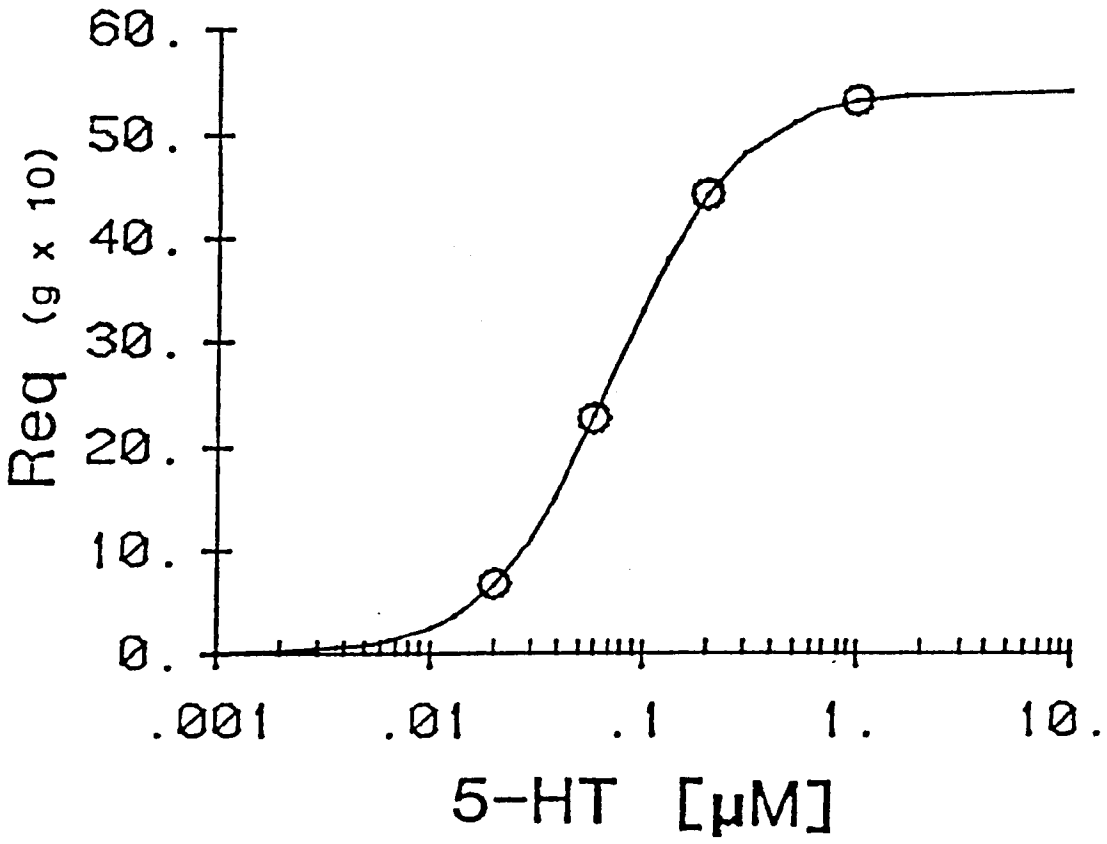

60.
50.
40.
30.
20.
10.
0.
Req (g x 10)
.001
.01
.1
1.
10.
5-HT [µM]

FIGURE 19: A concentration - response curve depicting the dependence of the equilibrium respone (R_{eq}) values of the tonic response on [NMT] in an individual aortic ring. The EC_{50} is 0.97 uM and the slope index is 1.13.

Req (g x 10)
0.
6.
12.
18.
24.
NMT [μM]
.01
.1
1.
10.
100.

FIGURE 20: The dependence of the observed rate constant of onset (k_{obs}) of the tonic response on [5-HT] in an individual aortic ring. The EC_{50} of this concentration - rate curve is 0.135 uM and the slope index is 1.59.

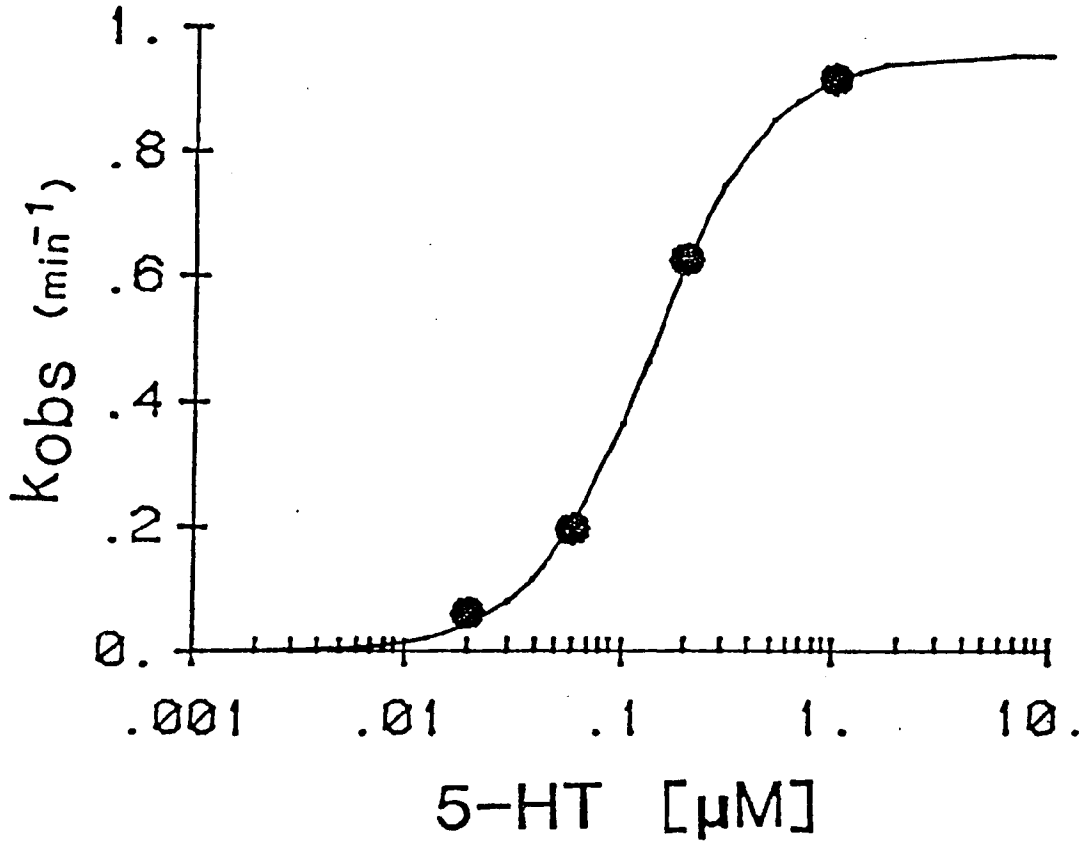

1.
.8
.6
.4
.2
0.
kobs (min⁻¹)
.001
.01
.1
1.
10.
5-HT [μM]

FIGURE 21: The dependence of the observed rate constant of onset (k_{obs}) of the tonic response on [NMT] in an individual aortic ring. The EC_{50} is 0.72 uM and the slope index is 1.24.

NMT [μM]
.01
.1
1.
10.
100.
0.
.2
.4
.6
.8
kobs (min⁻¹)

able function. The EC_{50} for k_{obs} vs. [5-HT] of 0.072 $\pm$ 0.024 uM (n = 4) is similar to the EC_{50} for Req vs. [5-HT]. The EC_{50} value for k_{obs} vs. [NMT] of 1.22 $\pm$ 0.35 uM (n = 4) is close to the EC_{50} value for R_{eq} vs. [NMT] (table 5).

TABLE 5

Summary of the characterization of the 5-HT and NMT Responses[a]

Drug		Phasic Response (R_0)		Tonic Response (R_{eq})	Tonic Response (k_{obs})	
5-HT	EC_{50} (uM)	0.22 ± 0.03	(5)	0.056 ± 0.011	0.072 ± 0.024	(4)
	slope index	1.37 ± 0.14		1.71 ± 0.22	1.57 ± 0.11	
NMT	EC_{50} (uM)	6.13 ± 1.07	(5)	0.79 ± 0.10	1.22 ± 0.35	(4)
	slope index	1.12 ± 0.18		1.45 ± 0.12	1.26 ± 0.13	

[a] Values are the mean ± S.E.M. of (n) experiments.

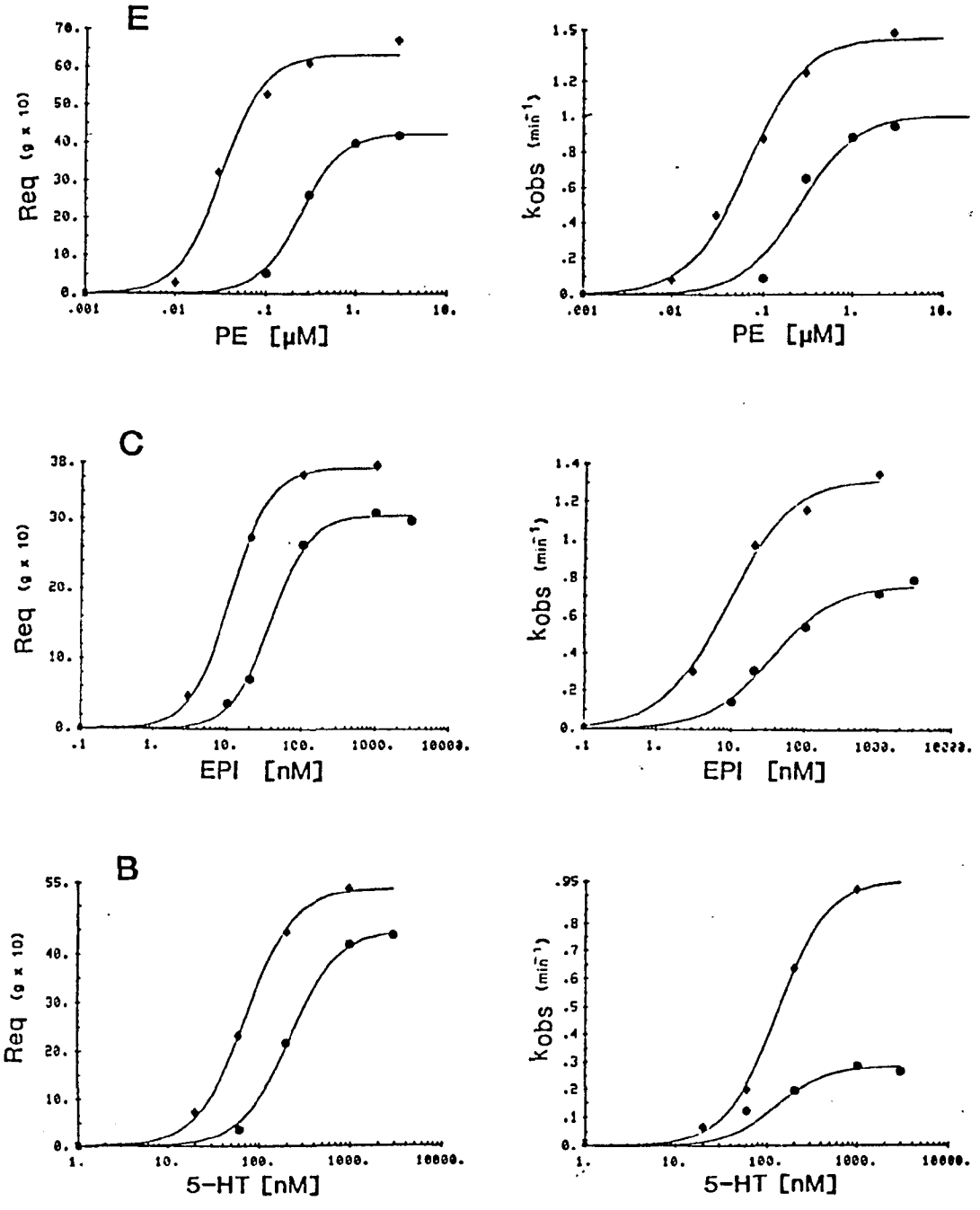
PART III. ALKYLATION STUDIES

The Effect of Receptor Alkylation on the Tonic Response to 5-HT, PE and EPI

Figures 20 and 21 show that k_{obs} reaches a limiting value (k_{obs}max) with increasing concentrations of 5-HT or NMT, respectively. The value of k_{obs} for any particular concentration of drug should depend on the amount of DR formed. If the number of free receptors is reduced the concentration - rate curve (k_{obs} vs. [drug]) should reflect this decrease in receptor number (according to equation 26). One method by which the amount of viable receptors in an isolated organ preparation can be reduced involves the use of a receptor selective alkylating agent. It was shown that the alkylating agent, dibenamine, selectively inactivates receptors over other constituents of the response generating system (Furchgott, 1966; Clancy and Maayani, 1984).

The dependence of k_{obs}max on the total number of receptors was investigated by assessing the concentration - rate relationships for three agonists before and after treatment with dibenamine. Figure 22 shows the effect of a 12 - 13 minute incubation with 0.5 uM dibenamine on the concentration - response and concentration - rate curves of three agonists. The concentration - response and concentration - rate curves of all three agonists are effec-

FIGURE 22: The effect of treatment of rabbit aorta rings with 0.5 uM dibenamine for 12 - 13 min on concentration - response (left panel) and the corresponding concentration - rate (right panel) curves to 5-HT, PE and EPI. The pre - treatment data points are indicated by diamonds, and the post - treatment data points by solid circles. Each row of curves is labeled with a capital letter to designate the corresponding experiment it represents in tables 6 and 7.


E
Req (g x 10)
70.
60.
50.
40.
30.
20.
10.
0.
.001
.01
.1
1.
10.
PE [μM]
kobs (min⁻¹)
1.5
1.2
1.
.8
.6
.4
.2
0.
.001
.01
.1
1.
10.
PE [μM]
C
Req (g x 10)
38.
30.
20.
10.
0.
.1
1.
10.
100.
1000.
10000.
EPI [nM]
kobs (min⁻¹)
1.4
1.2
1.
.8
.6
.4
.2
0.
.1
1.
10.
100.
1000.
EPI [nM]
B
Req (g x 10)
55.
40.
30.
20.
10.
0.
1.
10.
100.
1000.
10000.
5-HT [nM]
kobs (min⁻¹)
.95
.8
.7
.6
.5
.4
.3
.2
.1
0.
1.
10.
100.
1000.
10000.
5-HT [nM]

ted by dibenamine treatment. A comparison of the reduction in $R_{eq}max$ (table 6) and $k_{obs}max$ (table 7) reveals for 5-HT and epinephrine (EPI) that the $k_{obs}max$ is decreased more than the $R_{eq}max$; for PE the $R_{eq}max$ and $k_{obs}max$ were equally effected. As shown in table 8, dibenamine caused a dextral shift (indicated by an increased EC_{50}) in the concentration - response curves for 5-HT, EPI and one experiment with PE (less than a twofold shift in the EC_{50} is not considered to be a significant shift). The effect of dibenamine treatment on the EC_{50} of the concentration - rate curves to 5-HT, EPI, and PE was variable; the concentration - rate curves were shifted in some experiments and not in others.

Table 9 shows the values of K_A (the agonist dissociation constant) and q (the proportion of receptor population remaining after alkylation) obtained for 5-HT, EPI and PE in experiments A - F of table 6. These values were calculated using the method of Furchgott and Burstyn (1967) from both the concentration - response and concentration - rate data. The values of K_A from the concentration - responses data were similar for the two experiments with 5-HT and with EPI but were variable for the two PE experiments.

TABLE 6

Effect of Treatment with 0.5 uM Dibenamine for 12 - 13 min on the $R_{eq}max$ of 5-HT, EPI, and PE [a]

	5-HT		EPI		PE	
	A	B	C	D	E	F
control (g x 10)	32.4	53.4	37.2	53.6	62.3	28.5
alkyl.	16.8	44.4	30.4	32.1	42.0	8.7
% decrease in $R_{eq}max$	(48 %)	(17 %)	(18 %)	(40 %)	(33 %)	(69 %)

[a] The data from individual experiments is listed in columns labeled A - F.

TABLE 7

Effect of Treatment with 0.5 uM Dibenamine for 12 - 13 min on the $k_{obs}max$ of 5-HT, EPI, and PE [a]

	5-HT		EPI		PE	
	A	B	C	D	E	F
control (min^{-1})	1.05	0.95	1.32	1.17	1.45	0.94
alkyl.	0.30	0.28	0.76	0.51	1.00	0.33
% decrease in $k_{obs}max$	(71 %)	(71 %)	(42 %)	(56 %)	(31 %)	(65 %)

[a] The data from individual experiments is listed in columns labeled A - F.

TABLE 8

Effect of Treatment with 0.5 um Dibenamine for 12 - 13 min on the EC_{50} Values for the 5-HT, EPI, and PE Concentration - Response and Concentration - Rate Curves [a]

Parameter	5-HT		EPI		PE	
	A	B	C	D	E	F
	(control: concentration - response)					
EC_{50} (nM)	48	72	11	22	32	73
slope index [b]	1.4	1.6	1.7	1.4	1.9	1.3
	(post - alkylation: concentration - response)					
EC_{50} (nM)	298	217	38	96	245	84

continued on the next page

Table 8 continued

	5-HT		EPI		PE	
			(control: concentration - rate)			
EC_{50} (nM)	54	131	9	14	68	179
slope index [b]	1.8	1.6	1.0	1.3	1.3	0.8
			(post - alkylation: concentration - rate)			
EC_{50} (nM)	81	131	36	17	260	180

[a] Values from individual experiments are listed in columns labeled A - F. The label of the column refers to the same experiments listed in Table 7.

[b] The control and alkylation data were fitted to a common slope index using the fitting procedure FITFUN described in the Methods Section.

TABLE 9

Values of K_A (the agonist dissociation constant) and q (the proportion of receptor remaining after alkylation) Calculated from the Tonic Response Alkylation Data[a]

Parameter	5-HT		EPI			PE
	A	B	C	D	E	F
	(Concentration - Response)					
K_A (nM)	441	758	136	169	510	60
q	0.12	0.27	0.27	0.18	0.13	0.43
	(Concentration - Rate)					
K_A (nM)	45	82	73	14	600	282
q	0.48	0.55	0.14	0.49	0.22	0.22

[a] The method of Furchgott and Burstyn (1967) was used to calculate the K_A and q values from both the concentration - response and concentration - rate data. Values from individual experiments are listed in columns labeled A - F. The label of the column refers to the same experiments listed in Table 7.

The Effect of Receptor Alkylation on the Phasic Response to 5-HT and PE:

Repeated exposure of an aorta ring to dibenamine reduces the peak of the phasic response to 20 uM PE and 5-HT. Table 10 shows the data for the fitted parameters of phasic responses from 6 rings in 6 experiments before and after repeated incubation of the tissue with dibenamine. These results reveal that R_0 is consistently reduced by receptor alkylation; the effect of alkylation on k_{on} and k_{decay} does not exhibit a consistent pattern. It seems that the alkylation procedure has no effect on these kinetic parameters.

Table 10

Effect of Dibenamine Treatment on the Phasic Response to 20 uM 5-HT and PE[a]

	5-HT				PE		
	R_0 (g x 10)	k_{on} (min^{-1})	k_{decay}		R_0 (g x 10)	k_{on} (min^{-1})	k_{decay}
control	12.4	3.6	0.15	control	10.3	5.6	0.11
7 min	2.2	2.7	0.20	7 min	4.3	4.2	0.23
4 min	1.2	4.2	0.11	4 min	1.7	6.6	0.11
4 min	0.9	3.0	0.20	4 min	--	--	--
control	24.0	3.6	0.33	control	47.4	3.6	0.10
7 min	6.9	2.4	0.43	7 min	37.8	3.2	0.22
7 min	1.4	3.0	0.39	7 min	25.3	3.0	0.40
10 min	0.8	1.0	0.85	10 min	7.7	2.0	0.42
3 min	--	--	--	3 min	0.2	3.3	0.09

continued on the next page

Table 10 continued

	5-HT				PE		
	R_0 (g x 10)	k_{on} (min^{-1})	k_{decay} (min^{-1})		R_0 (g x 10)	k_{on} (min^{-1})	k_{decay} (min^{-1})
control	15.0	5.5	0.22	control	13.9	7.4	0.13
7 min[b]	8.0	5.8	0.51	12 min	5.9	5.6	0.33
4 min[b]	4.0	3.0	0.77	10 min	1.0	6.9	0.21

[a] The data from six experiments is shown.

[b] The incubation time with 0.1 uM Dibenamine.

All other incubations are with 0.5 uM Dibenamine.

PART IV. PARTIAL AGONIST STUDIES

The Dependence of the Kinetics of the Tonic Response on the Nature of the Agonist:

To test the dependence of the $k_{obs}max$ on the nature of the drug, the $k_{obs}max$ values of a series of serotonergic agonists were determined by measuring the k_{obs} of the tonic response to a saturating concentration (20 uM) of agonist. When the $k_{obs}max$ values of a series of drugs are determined in the same ring, R_{tot} is constant and so the relative $k_{obs}max$ values should be equivalent to the relative intrinsic rate constants (k_{int}) according to equation 26. A semilogarithimic plot of the response vs. time for a single experiment shows that the slopes (which are an estimate of $k_{obs}max$) of the responses increase in the order: DMT, NMT, Quip, Tryp, 5-HT (fig. 23). This rank order of $k_{obs}max$ values is in agreement with the rank order of the maximal response achieved by the drugs ($R_{eq}max$) (table 11). There is a good correspondence between the relative $R_{eq}max$ and relative $k_{obs}max$ for all drugs except 5-HT. The relative $k_{obs}max$ of 5-HT is noticeably greater than its relative $R_{eq}max$ value.

FIGURE 23: A semilogarithmic plot of the onset of the tonic responses to a saturating concentration (20 uM) of five serotonergic agonists acting on an individual aortic ring. The slopes of the fitted lines ($r^2 \geq 0.98$) provide estimates of the observed rate constant of onset (k_{obs}). The cumulative data for this experiment is presented in table 11.

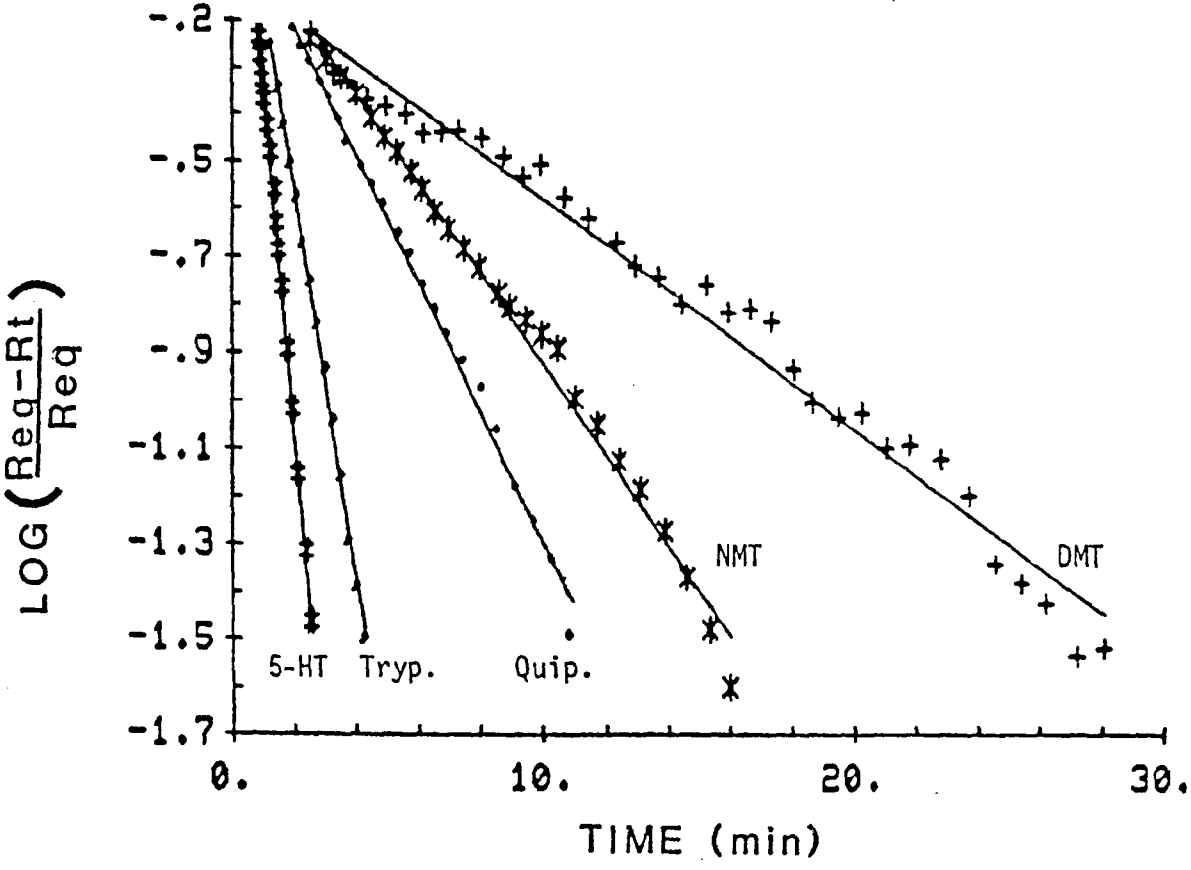

-.2
-.5
-.7
-.9
-1.1
-1.3
-1.5
-1.7
LOG (Req-Rt / Req)
0.
10.
20.
30.
TIME (min)
5-HT
Tryp.
Quip.
NMT
DMT

TABLE 11

Parameters of Tonic Responses to 20 uM of 5-HT Selective Agonists[a]

Drug	Relative R_{eq}max ± S.E.M.	Relative k_{obs}max ± S.E.M.	(n)
5-HT	2.92 ± 0.27	4.80 ± 0.64	(13)
Tryp	2.21 ± 0.28	2.45 ± 0.31	(7)
Quip	1.89 ± 0.25	2.16 ± 0.44	(4)
AMT	1.51 ± 0.20	1.41 ± 0.19	(3)
NMT	1	1	(13)
DMT	0.55 ± 0.06	0.68 ± 0.13	(7)

[a] Reqmax and kobsmax values are expressed relative to the value of NMT.

The Dependence of the Kinetics of the Phasic Response on the Nature of the Agonist:

The phasic responses to 5-HT and three tryptamine analogs were elicited with a saturating concentration of the drug and are shown in figure 24. The cumulative results for the parameters obtained from the fitting of the phasic responses of these drugs, DMT and quipazine to equation 21 are shown in table 12. The values of R_0 from each experiment were expressed relative to the value of NMT. The rank order of agonist intrinsic activities expressed as relative R_0 values in increasing order is: DMT, NMT, AMT, Quip, Tryp and 5-HT. This rank order of relative R_0 values is the same as the rank order of relative $k_{obs}max$ and $R_{eq}max$ values for these drugs (see table 11). Neither the rank order of relative k_{on} values nor relative k_{decay} values correspond to the rank order of relative R_0 values.

FIGURE 24: A characteristic kinetic profile of the phasic responses to a saturating concentration of (20 uM) of four serotonergic agonists acting on an individual aortic ring. These responses were fitted to equation 21. The cumulative data for the fitted parameters are presented in table 12.

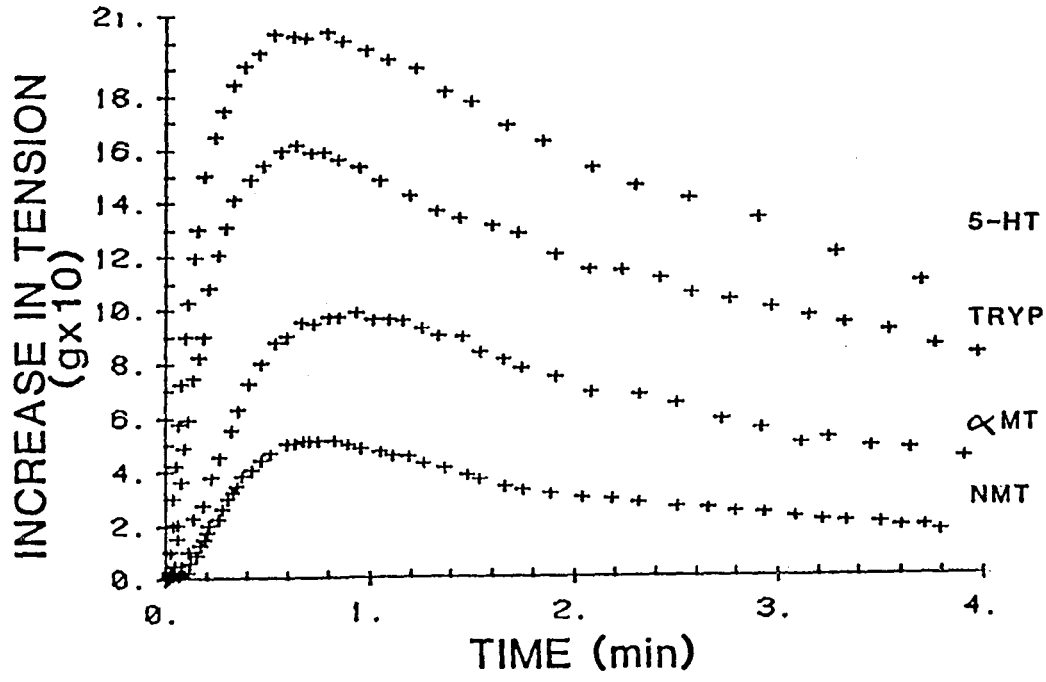

INCREASE IN TENSION
(gx10)
21.
18.
16.
14.
12.
10.
8.
6.
4.
2.
0.
0.
1.
2.
3.
4.
TIME (min)
5-HT
TRYP
αMT
NMT

TABLE 12

Parameters of Phasic Responses to 20 uM of 5-HT Selective Agonists[a]

Drug	R_0/R_0NMT ± S.E.M.	k_{on}/k_{on}NMT ± S.E.M.	k_{decay} ± S.E.M.	(n)
			(min^{-1})	
5-HT	4.46 ± 0.32	2.44 ± 0.36	0.20 ± 0.06	(3)
Tryp	2.93 ± 0.31	1.52 ± 0.09	0.13 ± 0.06	(2)
Quip	2.15	1.40	0.15	(1)
AMT	1.65 ± 0.08	1.46 ± 0.07	0.27 ± 0.04	(3)
NMT	1	1	0.30 ± 0.03	(3)
DMT	0.40 ± 0.10	1.89 ± 0.43	0.33 ± 0.06	(3)

[a] Values of R_0 and k_{on} in each experiment were expressed relative to the value of NMT.

DISCUSSION

Receptor - mediated biphasic contractions are a common phenomenon in smooth muscle preparations. It was shown that the biphasic nature of these responses arises from two processes which are differentially sensitive to changes in the extracellular calcium concentration. The phasic component is due to the release of stored intracellular calcium whereas the tonic component is generated by the influx of extracellular calcium (for a review see the Appendix). In some tissues the separation between the phasic and the tonic components of the response is well defined, e.g. in the rabbit ear artery (Steinsland et al., 1973) and in the guinea pig ileum (Triggle and Chang, 1973) contractions. In other preparations, there is not a clear distinction between the phasic component and the beginning of the tonic component. In particular, in the rabbit aorta, the tissue investigated in this work, it is virtually impossible to distinguish between the phasic and tonic components for the drug concentration range around the EC_{50}. Therefore a special method was developed to separate the two components and to enable the investigation of the pharmacological parameters of these processes by separately characterizing the steady state and kinetic properties of both processes.

The successful separation of the adrenergic (PE) and serotonergic (5-HT and NMT) agonist - induced contractions of the rabbit aorta into the two components (see Materials and Methods) and the kinetic analysis of each provides information about the mechanism of the drug - induced events that is unavailable from steady state studies. For example, the characterization of the concentration - response relationship of PE in the rabbit aorta derived from the steady - state levels of the total response yields essentially two parameters, i.e., EC_{50} = 0.2 uM and a slope index of 1.16 (fig. 3). These values do not indicate that the response is composed of two distinct components with different kinetic characteristics. These become evident only after the separation of the components. The characteristics of the two components are summarized in table 13.

The Phasic Response:

The phasic response for both the alpha-1 adrenergic and serotonergic receptor systems is independent of extracellular calcium and is characterized by a rapid onset that peaks within 1.5 minutes followed by a slower decay. The rate constant for the decay shows no dependence on either the concentration of drug or on the concentration of calcium in the preincubation buffer and is therefore a first order process. The onset however, is sensitive to the con-

TABLE 13

Characteristics of the phasic and tonic responses to PE

Response	Steady - State Properties	Kinetic Properties
Phasic[a]	R_0 shows a saturable dependence of [PE] (EC_{50} = 0.17 + 0.02 uM) and on preincubation [Ca++] (EC_{50} = 0.09 + 0.02 mM)	k_{on}: a) increases with [PE], but is not ostensibly saturable; and b) is independent of the preincubation [Ca++]. k_{decay} is first order and independent of [PE] and preincubation [Ca++].
Tonic[b]	R_{eq} shows a saturable dependence on [PE] (EC_{50} = 0.13 + 0.07 uM) and on [Ca++] (EC_{50} = 0.20 + 0.02 mM).	k_{obs}: a) shows a saturable dependence on [PE] (EC_{50} = 0.18 + 0.12 uM); a pseudo - first order dependence on [DR]; and b) a pseudo - first order dependence on [Ca++].

[a] For the definitions of R_0, k_{on} and k_{decay} see equation 21.

[b] For the definitions of R_{eq} and k_{obs} see equations 23 to 26.

centration of the drug. Because the phasic response is transient its steady state properties had to be characterized in terms of the maximal attainable response, R_0. This is the response the system would produce in the absence of decay. This property cannot be directly measured but is obtained from equation 21 which describes the essential behavior of the phasic response. R_0 is saturable with respect to drug concentration indicating that the phasic response is receptor - mediated. R_0 is also saturable with respect to the preincubation concentration of calcium (table 3). This corroborates the observation of van Breemen et al. (1972) that the phasic component relies on a limited source of intracellular calcium. The combined phenomena of a first order decay process and a limited source of intracellular calcium account for the transient nature of this response.

The dependence of the apparent rate constant of onset of the phasic response, k_{on}, on both the concentrations of drug and calcium differs from the dependence exhibited by the steady state parameter R_0. k_{on} <u>depends</u> on drug concentration in what appears to be a nonsaturable manner and does <u>not depend</u> on the concentration of calcium. Since the EC_{50} values predicted by the saturable model (equation 17) are so much greater than the EC_{50} of R_0 vs. drug concentration it was concluded that k_{on} does not depend on [DR]. The apparent nonsaturable dependence of k_{on} on drug

concentration suggests that the rate - determining step of the phasic response is the access of the drug to the receptor (i.e., the formation of DR). Given the accepted mechanism of the phasic response in which the formation of DR triggers the release of intracellular calcium (see review, Daniel et al., 1972; van Breemen et al., 1982, Appendix of this thesis), the lack of dependence of the kinetic parameters on the concentration of calcium suggests that the intracellular calcium stores are released at a rate exceeding the rate of formation of DR.

The Tonic Response:

The tonic response for both the alpha-1 adrenergic and serotonergic receptor systems reaches a steady state level of contraction (R_{eq}) that shows a saturable dependence on the concentrations of both the drug and extracellular calcium. The saturable dependence on drug concentration indicates that like the phasic response, the tonic response is receptor mediated. The EC_{50} of the tonic response parameter R_{eq} vs. [PE] is independent of the concentration of calcium in the buffer. This lack of dependence between PE and calcium suggests that calcium does not interact directly with the alpha-1 adrenergic receptor.

The dependence of R_{eq} on the extracellular calcium concentration demonstrates that extracellular calcium has an

integral role in the response generating process but the mechanistic implications of the saturable dependence are not clear. As discussed in the Appendix, many components of the smooth muscle contractile apparatus depend on calcium. Among the most important are calcium channels, calmodulin, and myosin light chain kinase (MLCK). It is impossible from the data presented in this thesis to determine which of these components is responsible for this saturable phenomenon.

An alternative physiological explanation of the saturability with respect to calcium concentration comes from the inhibitory effect that high calcium has on the contractile response. In agreement with Bohr (1963) I observed that high concentrations of calcium (above 5 mM) induce a depression of the contractile reponse. Bohr (1963) proposed that high calcium concentrations have a "membrane stabilizing effect" which inhibits the drug - stimulated release of intracellular calcium. This hypothesis refers to the depression of the phasic portion of the response observed by Bohr (1963) but does not explain the depression of the tonic response observed in this study. However, a functional model for this phenomenon is suggested by the behavior of membrane calcium channels. In some organisms, e.g. paramecium (Brehm et al., 1980) and aplysia (Tillotson, 1979; Eckeret and Tillotson, 1981), calcium channels are inactivated by a high concentration of intracellular calcium

that results from the influx of extracellular calcium. This could also be the explanation for the inhibitory effect of high calcium concentrations on the contractile response in the rabbit aorta. Thus, the inhibitory effect of high calcium combined with the normal dependence of the response on calcium may explain the apparent saturation of R_{eq} as calcium concentrations are increased.

The tonic response exhibits a first order rate of onset and therefore the apparent rate constant of onset (k_{obs}) was estimated from the slope of a semilogarithimic transformation of the onset phase (e.g. fig. 10). k_{obs} shows a saturable dependence on the concentration of drug and a linear dependence on the concentration of extracellular calcium. These rate constants reflect the dependence of the rate - determining step on the concentrations of drug and calcium. The saturability of k_{obs} with respect to the concentration of agonist suggests that this step is pseudo - first order with respect to DR. This implies that the rate - determining step occurs <u>after</u> the formation of DR and is linearly dependent on the concentration of DR. Pseudo - first order dependence is also observed with respect to calcium concentration in the range 0.2 - 2.22 mM indicating that calcium participates in the rate - determining step. Similar observations by Sunano and Miyazaki (1968) in the depolarized guinea pig taenia coli demonstrate that different smooth muscles may have a

similar dependence of the rate - determining step on calcium.

Differences in the Alpha-1 Adrenergic and Serotonergic Response Characteristics:

An important difference between the alpha-1 adrenergic and the serotonergic phasic responses is the appearance of a decreased response to high concentrations of the latter. This is apparent at the higher concentrations of 5-HT and NMT for which the values of R_0 (tables 3 and 4) are less than at lower concentrations. In contrast, the rate constant of onset of the phasic response, k_{on}, did not show desensitization to high concentrations of 5-HT or NMT. It is interesting to note that a parameter dependent on DR, (R_0) shows desensitization while k_{on}, a kinetic parameter that is independent of DR, does not. This observation suggests that the concentration dependent relaxation of the serotonergic contractile response of the rabbit aorta may be a receptor - mediated event.

Another difference between the adrenergic and serotonergic response characteristics is evident when comparing the EC_{50} value of the phasic response parameter R_0 and the EC_{50} value of the tonic response parameter R_{eq}. Whereas in the adrenergic system these values are similar (table 13), in the serotonergic system the EC_{50} values for

R_0 are greater than the EC_{50} values for R_{eq} (table 5). There are two possible explanations for these differences: i) either the phasic and tonic responses are elicited by the action of the agonist at two different 5-HT receptors, or ii) a single 5-HT receptor interacts with two different effector mechanisms to give rise to two responses.

The first possibility, i.e., that there are two receptors, cannot be excluded by the available 5-HT receptor classification studies. These studies measure the steady - state responses achieved during the tonic component of the normal biphasic response and so do not shed light on the receptor that mediates the phasic component. The strongest evidence against the involvement of two separate receptors in the biphasic response arises from $^{45}Ca^{++}$ flux studies in the rabbit aorta. Awad et al., (1983) demonstrated that both agonist - stimulated $^{45}Ca^{++}$ efflux from the rabbit aorta (a signal of the intracellular release of calcium during the phasic response) and $^{45}Ca^{++}$ uptake (corresponding to calcium influx during the tonic response) into the rabbit aorta are mediated by the same alpha-1 adrenergic receptor (see the Introduction section on Adrenergic Classification in the Rabbit Aorta). Thus a single receptor type mediates both the phasic and tonic components of the adrenergic response. The characteristics of the tonic and phasic responses to the 5-HT agonists and PE are qualitatively similar; it is therefore unlikely that the serotonergic

drugs elicit the two components of the contractile response through different receptors whereas the adrenergic drugs act through a single class of receptors.

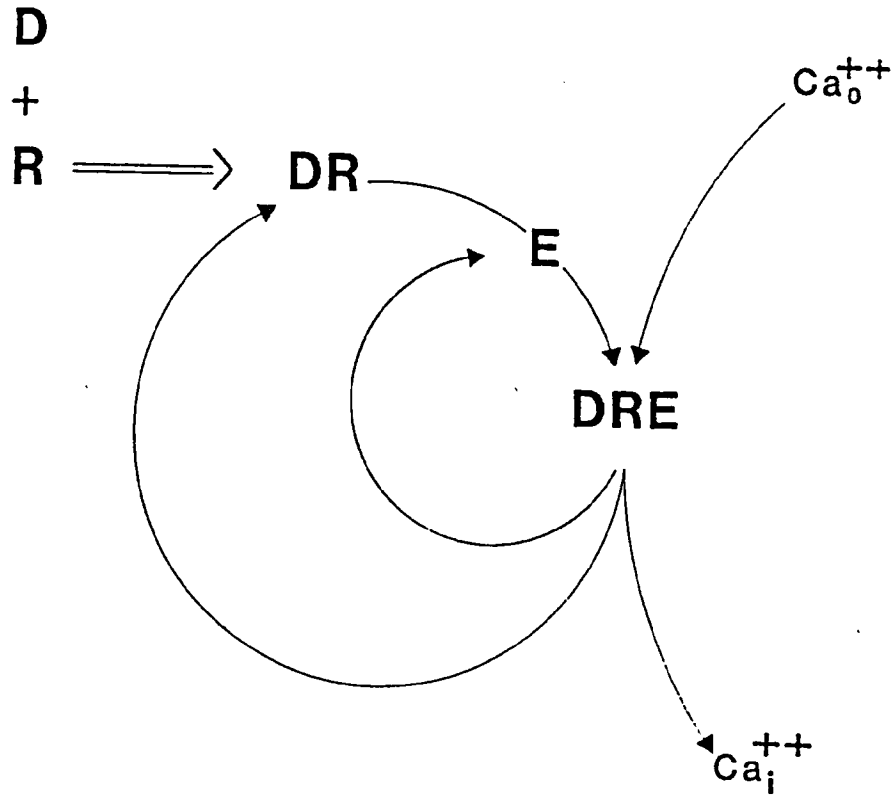
The different EC_{50} values for the R_0 and R_{eq} data can be explained by the fact that the phasic and tonic responses mobilize calcium in different ways and and from different sources (see Appendix). The phasic response is generated by the release of calcium from limited intracellular cellular sources whereas the tonic response is induced by the influx of calcium from an essentially infinite extracellular compartment. Thus, the same drug - receptor complex could initiate two separate responses by interacting with independent effectors: one mobilizing intracellular calcium and the other enabling the influx of extracellular calcium into the smooth muscle cell. The different EC_{50} values of R_0 and R_{eq} may reflect these different coupling events. This duality in effector activation mechanisms by a single receptor type should hold true in the adrenergic system as well. In this case the similarity of the EC_{50} values for R_0 and R_{eq} vs. [PE] may be regarded as coincidental.

The Proposed Model for the Generation of the Tonic Response:

The proposed mechanism of response generation in the rabbit aorta that emerges from these studies is as follows: the primary event is the interaction of the drug (D) with

the receptor (R) to form DR. DR initiates two events: the first is a rapid release of calcium from intracellular stores, the second is a slower influx of calcium into the cell. Both events trigger the contractile machinery and give rise, respectively, to the phasic and the tonic components of the response. Because the release of the intracellular calcium is faster than the formation of DR, the observed rate constant for the onset of the phasic response reflects the rate - determining step, the access of D to R, and therefore does not saturate with increasing concentrations of drug. The dependence of the rate constant of the second event (depicted in fig. 25) on drug concentration is consistent with a rapid formation of DR and a relatively slow interaction of DR with a hypothetical effector, E, and its activation in the form DRE. In this activated form the effector, which could be a calcium channel, enables the transport of calcium into the cell to produce the tonic component of the response. This mode of transport of calcium is consistent with the observed linear dependence of the rate constant for the onset of the tonic response on the concentration of calcium. The obligatory role for calcium in the generation of the tonic response and the dependence of k_{obs} on the calcium concentration suggests that calcium participates also in the formation of the activated effector DRE (fig. 25). In the context of the model, manipulating the concentration of extracellular cal-

FIGURE 25: A model depicting the rate - determining step leading to the generation of the tonic response in the rabbit aorta. The drug (D) and the receptor interact to produce DR. DR in turn interacts with the effector (E) to produce the activated effector, indicated by DRE, which enables the translocation of calcium from the outside to the inside of the smooth muscle cell. The contractile response is generated by this influx of extracellular calcium. In this model [DR] and [Ca^{++}_{o}] are in large excess over [E] so that the reaction rate constant will depend in a pseudo - first order manner on their concentrations.


D
+
R
DR
E
Ca_o^{++}
DRE
Ca_i^{++}

cium changes the ability of DR to couple to E. The decreased coupling of DR to E at low calcium concentration results in a decrease in the value of the intrinsic rate constant (k_{int}, see equation 26) which is reflected in the lower value of the measured rate constant, k_{obs}.

The observed approach of the tonic response to the steady - state level is the consequence of the equalization of the rates of two processes: the rate of calcium translocation into the cell and the rate of calcium elimination from the contractile machinery. From the observation that the phasic response decays in a first order process (fig. 6), i.e., the rate of decay is proportional to the size of the response, it can be inferred that the process of decay is associated with the elimination of calcium from the contractile part of the cell. Evidence for this process comes from studies in the rabbit aorta that demonstrate agonist - stimulated efflux of intracellular $^{45}Ca^{++}$ (van Breemen, 1969; van Breemen et al., 1982). The elimination of calcium may involve uptake of calcium into intracellular organelles (van Breemen, 1969; Mueller and van Breemen,1979) or efflux of calcium from the cell (Caroni and Carafoli, 1981). In the phasic response the calcium elimination process may be responsible for the observed decay of the response, due to the limited amount of calcium released from the intracellular pools. The elimination of calcium should occur during both the phasic and the tonic

portions of the response. But for the tonic response the elimination process results in a different behavior, i.e. the production of a steady state contraction. The rate of calcium translocation, therefore, should depend on two parameters: the amount of activated effector, DRE, and the concentration of extracellular calcium. The dependence on these parameters is indeed reflected in k_{obs}.

Within the framework of the model shown in figure 25 the concept of different efficacies for different drugs acting in this system on the same receptor acquires a new meaning. At a constant level of extracellular calcium the rate - determining step in the formation of the tonic response is the activation of the effector by DR. The observed rate constant for this process becomes equal to $k_{obs}max$ when the system reaches its maximal response at saturating conditions (i.e., $D >> K_D$ in equation 26). As explained above, the steady - state level of the response is the consequence of the equalization of two rate processes of which only one depends on DR. Thus, for a drug with a smaller $k_{obs}max$ the level at which it will reach steady - state will be lower than that for a drug with a larger $k_{obs}max$. This is consistent with the concept that the efficacy of a partial agonist is less than that of a full agonist. Furthermore, $k_{obs}max$ contains the two parameters that determine efficacy and explain partial agonism. These are related to the nature of the tissue and the properties

of the drug. The $k_{obs}max$ is the product of the total number of receptors, R_{tot}, and the intrinsic rate constant, k_{int}, which is equivalent to the intrinsic efficacy as formulated by Furchgott and Bursztyn (1967). The total number of receptors, a property of the tissue, can determine the value of $k_{obs}max$ (fig. 22, table 7) and thus affect the efficacy of the drug. The intrinsic rate constant, k_{int}, on the other hand reflects the intrinsic efficacy of the activation of the effector by DR. Therefore it implicitly depends on the properties of the drug and its ability to activate the effector through the drug - receptor complex.

The Effect of a Reduction in R_{tot} on the Phasic and Tonic Responses

The alkylation studies provide evidence that $k_{obs}max$ depends on R_{tot}. A reduction in the maximal tonic response ($R_{eq}max$) is accompanied by a reduction in $k_{obs}max$ (tables 6, 7 and fig. 22). As discussed above $k_{obs}max$ should depend linearly on the number of receptors but within the experimental scope of this thesis it is not possible to verify this dependence directly. Furchgott and Burstyn (1967), in their discussion of the alkylation method for estimating agonist dissociation constants (K_A) assume that there is a linear relationship between receptor occupancy and stimulus. This assumption is supported by the finding

that the same value of K_A is obtained when the receptor population is alkylated to different degrees. The technique employed here to measure the response kinetics enables the construction of only a single control and post - alkylation concentration - rate curve for each aortic ring. However, when different experiments (table 9) were analyzed by Furchgott's method (1967) similar K_A values, (except for one experiment with PE), can be obtained for the same agonist after different degrees of alkylation (shown by the different q values). It appears then, that the relationship between $k_{obs}max$, a measure of the drug's stimulus properties, and R_{tot} may also be linear.

The phasic response is also sensitive to a reduction of receptor number by alkylation. Table 10 shows that progressive alkylation reduces R_0 . The rate constant of onset, k_{on}, in contrast is independent of the reduction of R_{tot}. These results indicate that while R_0 depends on the number of viable receptors k_{on} does not. The lack of dependence of k_{on} on the receptor is in agreement with the conclusion that this rate constant reflects the formation of DR, as evidenced by the nonsaturability of k_{on} with respect to drug concentration.

The Dependence of the Phasic and Tonic Responses on the Nature of the Drug

The R_0 values of the phasic response exhibit a dependence on the nature of the agonist. As seen in tables 11 and 12 the rank order of R_0 values for a group of 5-HT receptor agonists parallels their rank order of $R_{eq}max$ and $k_{obs}max$ values. Thus, R_0, is sensitive to the different stimulus properties of these drugs. k_{on}, to the contrary, shows no obvious dependence on the type of agonist. This lack of dependence lends further support to the assertion that the value of k_{on} is not determined by a post - receptor event.

The availability of a series of agonists acting at the 5-HT receptor with different efficacies enabled me to compare their stimulus properties. By comparing values of k_{obs} obtained from individual aortic rings the effect of differences in R_{tot} between tissues is avoided. Under these conditions the relative $k_{obs}max$ is equal to the relative intrinsic rate constant, k_{int} (equation 26). Table 11 shows that the values of relative $R_{eq}max$ and relative $k_{obs}max$ values are in good agreement for all the agonists except 5-HT. This discrepancy in the values of the relative parameters for 5-HT suggests that, although they are both sensitive to the nature of the agonist, $k_{obs}max$ and $R_{eq}max$ reflect different properties of the agonist - induced

response. The relative $R_{eq}max$ values shown in table 14 are the intrinsic activities defined by Ariens (1964) and therefore are not satisfactory measurements of the stimulus properties of drugs (see Introduction section: Evolution of Receptor Theory). $k_{obs}max$, on the other hand, is a direct measure of the stimulus properties of a drug. Therefore a comparison of relative $k_{obs}max$ values for different drugs acting on the same receptor is a comparison of the relative stimulus properties of these drugs. Table 14 shows the $k_{obs}max$ values for these agonists expressed relative to the value of 5-HT. The results in this table are derived from the same data presented in table 11 in which the values of $kobs_{max}$ and $R_{eq}max$ were expressed relative to the NMT value. The values of relative $k_{obs}max$ for these agonists are, in good agreement with the values of relative efficacy determined from steady state measurements (table 14).

Values of efficacy determined from steady state measurements must be expressed in relative form. But $k_{obs}max$ is the rate constant of the rate - determining step in response generation and therefore need not be expressed in relative form. If the number of receptors, R_{tot} in a specific preparation could be determined, e.g. by radioligand binding techniques, the value of the intrinsic rate constant, k_{int}, for any drug could be obtained from its $k_{obs}max$ value (equation 26). Thus, the kinetic characterization of the rate - determining step in the gen-

TABLE 14

Parameters of the Tonic Responses and the Efficacies of 5-HT Selective Agonists[a]

Drug	Relative $R_{eq}max$[b]	Relative $k_{obs}max$[b]	Relative Efficacy[b]
5-HT	1	1	1
Tryp	0.80 ± 0.06	0.63 ± 0.03	0.61 ± 0.09[c]
Quip	0.75 ± 0.04	0.44 ± 0.06	0.55 ± 0.06[e]
AMT	0.65 ± 0.07	0.37 ± 0.07	0.42 ± 0.06[c]
NMT	0.38 ± 0.04	0.24 ± 0.02	0.21 ± 0.01[d]
DMT	0.20 ± 0.03	0.18 ± 0.05	0.23 ± 0.03[e]

[a] $R_{eq}max$ and $k_{obs}max$ values are derived from the same data presented in table 11. A saturating concentration (20 uM) of the agonists was used.

Here they are expressed relative to the value of 5-HT. See table 11 for the number of experiments (n) for each drug.

[b] Mean ± S.E.M.

[c] Relative efficacy determined by Clancy and Maayani (1984) using the alkylation technique developed by Furchgott and Burstyn (1967).

[d] Relative efficay determined from the data of Clancy (personal communication) using the method of Barlow et al., (1967).

[e] Relative efficacy is the average value from two experiments and was determined by the method of Barlow et al., (1967) as described in the Method Section of this thesis.

eration of the contractile response can provide a direct measurement of intrinsic efficacy.

Summary:

Kinetic analysis of a receptor mediated contraction in a smooth muscle was used to elucidate the mechanisms of response generation. The rabbit aorta contractile response to a group of agonists was separated into a phasic and tonic response by virtue of their different dependence on the concentration of calcium in the buffer. Only a kinetic analysis of the response data revealed that there are different rate - determining steps for these two responses. The phasic response is probably a diffusion - limited process while the rate - determining step in the tonic response is the activation of an effector by the DR complex which enables the translocation of calcium into the cell. The kinetic scheme consistent with these observations predicts that the efficacy of a drug is the maximal rate constant of activation of the effector by the DR complex. It was shown that this efficacy term ($k_{obs}max$) depends on both the total number of receptors (R_{tot}) and on the nature of the drug. According to the model proposed in the thesis, $k_{obs}max$ can be written as a product of R_{tot} and the intrinsic efficacy constant (k_{int}). Since k_{int} is the rate constant for the activation of an effector by a drug -

receptor complex it should depend on the drug as well as the effector activated by the drug - receptor complex. Consequently, the intrinsic efficacy of a drug as measured by k_{int} will be different for responses elicited by the same receptor but mediated through different effector mechanisms.

APPENDIX

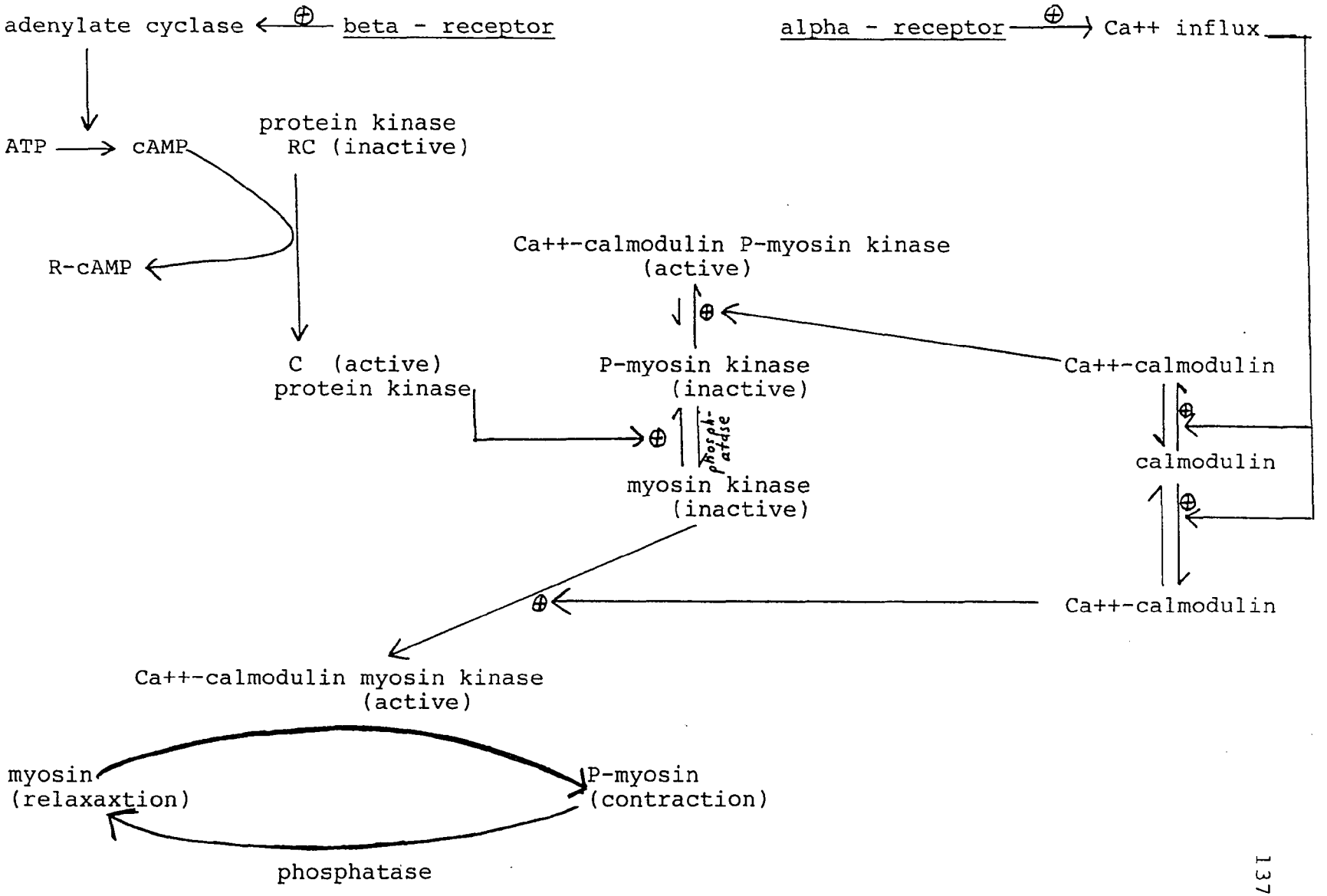
THE ROLE OF CALCIUM IN THE CONTRACTION OF SMOOTH MUSCLE

Introduction:

An overview of a currently acceptable model for the regulation of smooth muscle contraction is depicted in figure 26 (adapted from Adelstein et al., 1981) and described below.

The action of agonists at two classes of adrenergic receptors, alpha and beta, can result in opposing effects on the contractile apparatus. Agonist stimulation of the alpha receptor mobilizes calcium from intracellular and extracellular compartments. Extracellular calcium enters the cell via calcium channels while intracellular calcium is released from a membrane bound pool. As a consequence of these two processes the intracellular free calcium concentration is raised from 0.1 uM to 1 - 10 uM. This rise in the level of intracellular calcium leads to increased binding of calcium to calmodulin. The calcium - calmodulin complex in turn binds to and activates the enzyme, myosin light chain kinase (MLCK). The activated MLCK can then phosphorylate the light chains of myosin. After phosphorylation by MLCK, myosin will interact with actin to produce a contraction. The phosphorylated myosin is dephos-

FIGURE 26: A currently acceptable model for the regulation of smooth muscle contraction. See text for a description.


adenylate cyclase
beta - receptor
alpha - receptor
Ca++ influx
ATP
cAMP
protein kinase
RC (inactive)
R-cAMP
Ca++-calmodulin P-myosin kinase
(active)
C (active)
protein kinase
P-myosin kinase
(inactive)
Ca++-calmodulin
phosphatase
calmodulin
myosin kinase
(inactive)
Ca++-calmodulin
Ca++-calmodulin myosin kinase
(active)
myosin
(relaxaxtion)
P-myosin
(contraction)
phosphatase

phorylated by myosin phosphatase. If the level of intracellular calcium is reduced, either by sequestration into intracellular organelles or by extrusion from the cell by ATPase pumps, the myosin dephosphorylating action of the phosphatase will result in relaxation of the contractile apparatus.

The alpha - adrenergic mediated contraction can be functionally antagonized by agonists acting on the beta - adrenergic receptor. The beta receptor - agonist complex stimulates adenylate cyclase to convert ATP to cAMP. The cAMP binds to the regulatory subunit of a protein kinase, freeing the catalytic subunit to phosphorylate the enzyme MLCK. The phosphorylation of MLCK brings it into an inactive state which binds poorly to Ca++ - calmodulin. Thus, by promoting the inactivation of MLCK, the beta receptor opposes the alpha receptor - mediated stimulation of MLCK.

In the review below aspects of the contractile process that relate specifically to the involvement of calcium will be discussed in more detail.

Calcium Channels:

The plasma membrane separates a millimolar concentration of extracellular calcium from a micromolar concentration of intracellular calcium. As a result of calcium influx the intracellular calcium concentration may increase by one or two orders of magnitude. The influx of calcium is mediated by voltage operated channels (VOCs) and possibly by receptor operated channels (ROCs) (Bolton, 1979; Cavero and Spedding, 1983). While VOCs have been well studied in excitable tissues, there is little known about ROCs.

The precise structure of the VOC is not known but it is thought to be a hydrophilic lipoprotein that preferentially permits the passage of calcium ions through the membrane (Cavero and Spedding, 1983; Reuter, 1983). Voltage and patch clamp studies reveal that during membrane depolarization the VOCs undergo activation and inactivation (Tsein, 1983). The process of channel activation is indicated by an increase in calcium conductance across the membrane while the process of inactivation is signified by a decrease in calcium conductance. Studies of cardiac muscle reveal that the activation time constant (5-20 msec) and inactivation time constant (30-300 msec) of calcium channels are slower than the corresponding processes of the voltage

operated sodium channel which has an activation time constant of about 1 msec and an inactvation time constant of 2-10 msec (Gettes, 1976; Katz et al., 1982). Consequently, sodium channels are referred to as 'fast channels' and calcium channels as 'slow channels'.

The activation and inactivation of a calcium channel is thought to occur via the action of voltage dependent gates contained within the channel. The gates are imagined to be charged residues lining the channel pore that are sensitive to the state of membrane polarization. Upon depolarization, gates open to allow the influx of calcium down its concentration gradient. Eventually a population of gates close to slow the passage of ions (Cavero and Spedding, 1983). The mechanism of inactivation of the channels is a matter of controversy. Depending on the tissue studied inactivation may occur in a voltage dependent manner or as a function of intracellular calcium concentration. Voltage dependent inactivation occurs in response to the state of membrane depolarization (Barrett and Barrett, 1976) and is described by the Hodgkin and Huxley model of sodium channel behavior in excitable tissue (Isenberg, 1977). An alternative mode of calcium channel inactivation results from the impedance of channel conductance by the accumulation of a high concentration of intracellular calcium following activation (Tsien, 1983). Voltage clamp experiments indicate that calcium channels of paramecium

(Brehm et al., 1980), aplysia (Tillotson, 1979; Eckert and Tillotson, 1981), stick insect (Ashcroft and Stanfield, 1982), and the snail, Helix aspersa, (Standen, 1981) inactivate by the latter method.

Intermittent between channel inactivation and activation is an interval of reactivation. The reactivation time for calcim VOCs (30-300 msec) places the channel in a refractory state while it resets for the next opening (Cavero and Spedding, 1983). No mechanistic interpretation of the reactivation was proposed.

Receptor operated channels are another vehicle by which calcium may enter the smooth muscle cell (Bolton, 1979). It is envisaged that an agonist - receptor complex directly or indirectly activates the channel. This type of activation is difficult to study experimentally because it may also lead to the activation of the VOCs (Cavero and Spedding, 1983) and the measurement of calcium currents from VOCs cannot be separated from those medaited by ROCS. Until selective antagonists of ROCs are found it will be difficult to distinguish ROCs from VOCs. The direct identification of calcium channels with radiolabelled calcium antagonists (Glossmann et al., 1982; Murphy et al., 1983) may provide insight into the structural differences between the two types of channels.

ROCs and PI Turnover:

Presently there is little understood about how agonists induce extracellular calcium mobilization. Presumably, the agonist - receptor complex somehow activates ROCs which allow the influx of calcium (Bolton, 1979). The nature of the channel - receptor interaction and the structure of the channel are unknown but recent advances in the field of phospholipid metabolism may provide insight into these mechanisms.

ROCs may not be stable protein structures as VOCs are thought to be but could be labile phospholipids which spontaneously form ionophores in the plasma membrane (Tyson et al., 1976; Putney et al., 1980). In many tissues, including smooth muscle (Salmon and Honeyman, 1980), the coexistence of agonist stimulated calcium flux and phosphatidylinositol (PI) turnover has been demonstrated. This correlation led Michell (1975) to postulate that these two processes are causally linked. In addition, the observation by Michell et al. (1981) that PI turnover in the hepatocyte is independent of external calcium suggests that PI turnover is not a consequence of calcium mobilization and may in fact precede it. Phamacological studies of PI turnover suggest that an as yet unidentified second messenger is produced prior to the PI cycle activation. The identification of a second messenger would serve to confirm

that stimulation of PI turnover is an event closely linked to the initial agonist - receptor interaction step (Billah and Michell, 1979, Kirk et al., 1981). The principal obstacle to establishing the role of PI turnover as the activator of calcium influx is the absence of a demonstrable link between these two phenomena. This difficulty may be related to the unstable nature of ROCs. Putney et al. (1980) observed that one product of PI turnover, phosphatidic acid (PA), may act as a calcium ionophore. They showed that in dispersed parotid cells tha addition of PA facilitated calcium uptake into the cells. It was suggested that endogenously produced PA may serve the same function in vivo. It is conceivable then that in vivo PA would behave as a ROC. If this were true the ROC would be a transient phenomenon, difficult to measure with experimental techniques, e.g. radioligand binding, that can identify only stable structures.

Mobilization of Calcium from Extracellular and Intracellular Sources:

In 1963 Bohr observed that the two phases of the rabbit aorta contractile response were differentially sensitive to changes in the concentration of calcium in the medium. Since then similar observations have been reported for a variety of smooth muscle preparations (Steinsland et al.,

1973; Chang and Triggle, 1973; Nelson and Tooke, 1974; Allen et al., 1976; Mukai and Kubota, 1980).

The biphasic response is composed of an initial fast response which is resistant to a lowering of extracellular calcium and a subsequent slow response that is sensitive to the level of extracellular calcium (Brodie et al., 1959; Bohr, 1963; Deth and van Breemen, 1974; 1974). Smooth muscle contraction is thought to depend on two operationally independent sources of calcium. In accordance with this idea, the two phases of the observed contraction represent the utilization of different calcium sources. The evidence for this proposal comes from two types of experiments. In one type of experiment agonist stimulated calcium flux is measured directly using either a radioactive calcium isotope or a calcium fluorescent indicator. The second type of experiment assesses the role of calcium in the contractile process by characterizing the dependence of the contraction on the level of extracellular calcium. Together these two approaches provide convincing evidence for the involvement of both an extracellular and an intracellular calcium pool in the biphasic contractile response. Except where otherwise indicated the work discussed below was done with the isolated rabbit aorta. In general, similar results are found for other smooth muscle preparations.

A) The effects of calcium - free medium

Agonists induce phasic contractile responses in calcium free medium (Karaki et al., 1979; Cory et al., 1984). Repeated response elicitation in calcium - free medium leads to a decrement of the phasic response. When extracellular calcium is restored to the medium and then, a few minutes later, the agonist is administered, the biphasic response reappears (Cory et al., 1984). These results support the assertion that there is a limited pool of intracellular calcium. When this limited pool is exhausted after repeated response elicitation in the calcium - free medium the phasic response cannot be generated. A short period of incubation in a medium with 2.22 mM calcium is sufficient to allow the internal calcium pool to refill (Karaki et al., 1979; Loutzenhiser and van Breemen, 1983, Cory et al., 1984).

B) Calcium antagonists

Drugs and ions which were shown to block the influx of calcium through calcium channels, eg. verapamil (Salaices et al., 1983), manganese (Keene et al., 1979; Deth and van Breemen, 1981), D-600 (Hester et al., 1979; Meisheri et al., 1981; Auguet and DeFeudis, 1982, Webb, 1982), lanthanum (van Breemen, et al., 1972; 1982), cinnarizine (Godfraind and

Kaba, 1969), and diltiazem (van Breemen et al., 1981; Saida and van Breemen, 1983), antagonize the slow phase of the response. At high concentrations calcium antagonists may block the slow phase of the response entirely and only a phasic response is observed. The effects of these drugs are generally reversible, i.e., after they are removed from the medium the biphasic reponse can again be elicited. Some agents like lanthanum are irreversible blockers of the slow phase (van Breemen et al., 1972; Karaki and Weiss, 1979; Deth and van Breemen, et al.; 1981). After administering lanthanum only one or two phasic responses can be elicited and then the tissue ceases to respond to agonist stimulation. This has been interpreted to mean that lanthanum, by blocking the entrance of calcium into the cell, prevents the refilling of the intracellular calcium pool used to generate the phasic response (van Breemen et al., 1972). This effect of lanthanum points to the necessity of refilling the intracellular calcium pool from an extracellular source before the phasic response can be elicited.

C) Radiolabelled calcium flux

The most compelling evidence for the existence of two sources of agonist stimulated calcium flux derives from studies with $^{45}Ca^{++}$. Using this technique both the agonist

induced uptake and release of calcium can be measured. Studies of agonist induced uptake of $^{45}Ca^{++}$ into rabbit aorta rings demonstrate the existence of receptor stimulated influx into the cell (van Breemen et al., 1982). The evidence for agonist induced release of intracellular calcium is based on a method which indirectly measures the agonist stimulated release of bound intracellular $^{45}Ca^{++}$ in tissues preincubated with the radiolabelled ion (van Breemen, 1969; van Breemen et al., 1982). In this method the tissue is incubated in $^{45}Ca^{++}$ to allow the cells to incorporate the isotope into the intracellular pool. The tissue is then washed in calcium - free buffer to remove extracellular calcium. Spontaneous efflux of intracellular $^{45}Ca^{++}$ is measured by collecting aliquots of the extracellular medium. Upon the addition of the agonist, an accelerated efflux of $^{45}Ca^{++}$ is observed. This increase in $^{45}Ca^{++}$ efflux above the basal rate is attributed to the rise in free intracellular calcium following the agonist stimulated release of bound intracellular calcium.

An early problem in the assessment of calcium efflux was the dilution of the $^{45}Ca^{++}$ by excess extracellular bound calcium that could not be removed by simply washing the tissue in calcium - free buffer. This problem was addressed with the development of the lanthanum technique by van Breemen (van Breemen et al., 1972), which involves

displacing the extracellular bound calcium with lanthanum prior to preincubation of the tissue in $^{45}Ca^{++}$.

The calcium flux studies revealed that agonists such as norepinephrine (van Breemen et al., 1982) and histamine (Hudgins, 1969) induce uptake of $^{45}Ca^{++}$ that can be blocked by calcium antagonists (Saida and van Breemen, 1983). Using the lanthanum technique to measure the agonist stimulated release of intracellular calcium it was shown that agonists acting on different receptors release a common store of intracellular calcium (Deth and Casteels, 1977).

D) Fluorescent indicators of calcium

This novel technique allows for the direct measurement of changes in extracellular calcium. The method utilizes a fluorsecent indicator of ionized calcium. The indicator permeates the cell membrane and is trapped in the cytoplasm after undergoing hydrolysis by intracellular enzymes. When intracellular free calcium is increased, by agonist induced release of intracellularly bound calcium or calcium influx into the cytoplasm, the indicator is excited and emits a measurable change in fluorescence (Tsien, 1980). One such indicator, Quin 2, was used to study the source of increased intracellular calcium involved in prolactin secretion from thyrotroin - releasing hormone (TRH) stimulated GH3 cultured cells (Gershengorn and Thaw, 1983). Using this indicator it

was determined that a TRH - induced increase in free intracellular calcium utilizes cellular calcium stores and that the influx of extracellular calcium is not a prerequisite to the observed increase in intracellular calcium. The application of this technique to smooth muscle will be helpful in determining the roles of intracellular calcium release and extracellular calcium influx in the contractile process.

Regulation of Contraction in Smooth Muscle:

The smooth muscle contractile apparatus is fundamentally similar to the sliding filament mechanism operative in skeletal and cardiac muscle. Smooth muscle contains both thin filaments (actin) and thick filaments (myosin) (Hartshorne and Gorecka, 1980). Tension is generated by the attachment of the globular head - light chain of the myosin filaments to receptive sites on actin, the so called cross bridge interaction (Hartshorne, 1982). Attachment of the myosin molecule to actin results in a shift in the angle of the attachment site so that after detachment the myosin can reattach to a second actin filament (Adelstein et al., 1981). Using this method of cyclic attachment, detachment and reattachment the two filaments slide past each other and the cell contracts. The interaction of actin and myosin feeds on the energy released

by the hydrolysis of Mg++ATP by Mg++ATPase (Adelstein et al., 1981. In addition to actin and myosin there exists another component of the contractile apparatus which serves a regulatory role. In skeletal and cardiac muscle this is called the tropomyosin - troponin complex. In a relaxed muscle tropomyosin protects the actin attachment sites from myosin binding. When intracellular calcium levels rise the calcium binds to the troponin C subunit causing it to weaken the interaction of actin with the troponin I subunit resulting in a movement of tropomyosin away from the actin attachment sites. These attachment sites can then interact with the myosin light chains. When intracellular calcium levels decrease, calcium dissociates from troponin C, the troponin - tropomyosin complex moves back over the actin attachment sites and the contractile apparatus relaxes (Hartshorne and Gorecka, 1980).

In smooth muscle there is no evidence for the existence of a troponin - tropomyosin complex but there is a troponin C - like molecule called calmodulin. In order for contraction to occur the myosin light chains must be phosphorylated by myosin light chain kinase (MLCK). When intracellular calcium is raised, calcium binds to calmodulin to form a Ca++ - calmodulin complex. This complex then binds to and activates MLCK (Kerrick et al., 1980; Mrwa and Hartshorne, 1980; Adelstein et al., 1981; Demaille et al., 1983).

Evidence for the direct effect of calmodulin on contraction comes from studies on skinned muscle preparation in which both contraction and myosin phosphorylation is measured. The skinning of a muscle fiber by mechanical (Hoar et al., 1979; Cassidy et al., 1981) or chemical (Gordon, 1978) means, removes the sarcolemma rendering the tissue highly permeable to ions and substrates. Using this preparation it was shown that exogenously added calmodulin, in the presence of a suprathreshold concentration of calcium, potentiates the action of calcium as indicated by a leftward shift of [Ca++] - contraction curves (Cassidy, 1979; Sparrow, 1981). MLCK mediated phosphorylation of myosin was demonstrated using an irreversible phosphorylating agent. After incubating skinned muscle tissue with ^{35}[S] - ATP it was shown that myosin had been thiophosphorylated and that this irreversible phosphorylation was accompanied by an irreversible increase in tension (Cassidy et al., 1979; Hoar et al., 1979). When a reversible phosphorylating substrate is used, e.g. ^{32}[P] - ATP, the phosphorylation is reversed by the action of the endogenous phosphatase (Adelstein et al., 1981). Thus, the relative activities of MLCK, which phosphorylates myosin, and phosphatase, which dephosphorylates myosin determine the level of tension in the smooth muscle cell.

Recently it was demonstrated that cAMP and calcium work in opposition in the smooth muscle contractile process

(Adelstein et al., 1981; Kerrick and Hoar, 1981; Schulman, 1982; Demaille et al., 1983); cAMP produced by beta - adrenergic receptor stimulation of adenylate cyclase may directly regulate the activity of MLCK. cAMP activates a cAMP - dependent protein kinase to phosphorylate MLCK. Phosphorylation of MLCK by protein kinase reduces its activity profoundly (Schulman, 1982; Demaille et al., 1983). This model for cAMP regulation of MLCK is supported by the results of Kerrick and Hoar (1981) who showed that the application of cAMP - dependent protein kinase catalytic subunit inhibits calcium activated tension in a skinned smooth muscle preparation. The regulatory roles of calcium and cAMP may not be independent of each other. It is believed, for example, that calcium may regulate cAMP levels by modulating the activity of phosphodiesterase (Schulman, 1982).

It therefore appears that there exist at least two mechanisms for inducing relaxation in smooth muscle; i) a lowering of free intracellular calcium below 0.1 uM by either, sequestration of calcium into cellular organelles (van Breemen, 1969; Mueller and van Breemen, 1979), or extrusion from the cell by pumps, e.g. Ca++ - ATPase or Na+/Ca++ ATPase (Caroni and Carafoli, 1981), and ii) a cAMP mediated relaxation. The lowering of free intracellular calcium causes a dissociation of the Ca++ - calmodulin complex. The affinity of calmodulin for MLCK is reduced in

the absence of calcium and so calmodulin will not associate with MLCK. Without Ca++ - calmodulin binding to it, MLCK cannot continue to phosphorylate myosin and thus the phosphatase will remove the remaining phosphates from myosin to bring about relaxation of the contractile apparatus. The second method of relaxation involves the cAMP dependent protein kinase. The kinase phosphorylates MLCK resulting in deactivation of the enzyme. Phosphatase will then cause relaxation by dephosphorylating myosin.

Conclusion:

Calcium mediates smooth muscle contraction in a complex manner. The entrance of calcium into the cell is regulated by the opening and closing of voltage operated and/or receptor operated channels. Agonist stimulated contraction of most smooth muscles depends on both the influx of extracellular calcium and the release of an intracellular pool of calcium. The intracellular concentration of calcium can regulate contraction in at least two ways, i) by directly stimulating the contractile apparatus via a Ca++ - calmodulin - MLCK complex and, ii) by modulating the cAMP - mediated relaxation effect via the calcium sensitivity of phosphodiesterase. An interesting area of future research will be the characterization of this functional antagonism between cAMP and calcium mediated effects. There appear to

be multiple loci of interaction between these two cellular regulators. Their opposing effects at these loci may determine the physiological state of the contractile elements in the smooth muscle cell.

REFERENCES

Adelstein, R.S., Pato, M.D. and Conti, M.A.: The role of phosphorylation in regulating contractile proteins. Advances in Cyclic Nucleotide Research. 14: 361-373, 1981.

Allen, G.S., Gross, C.J., Henderson, L.M. and Chou, S.N.: Cerebral arterial spasm, Part 4: In vitro effects of temperature, serotonin analogues, large nonphysiological concentrations of serotonin, and extracellular calcium and magnesium on serotonin - induced contractions of the canine basilar artery. J. Neurosurg.. 44: 585-593, 1976.

Apperley, E., Humphrey, P.P.P. and Levy, G.P.: Receptors for 5-hydroxytryptamine and noradrenaline in rabbit isolated ear artery aorta. Br. J. Pharmac.. 58: 211-221, 1976.

Ariens, E.J.: Affinity and intrinsic activity in the theory of competitive inhibition. Archs. Int. Pharmacodyn. Ther.. 99: 32-49, 1954.

Ashcroft, F.M. and Stanfield, P.R.: Calcium inactivation in skeletal muscle fibres of the stick insect, Carausius Morosus. J. Physiol.. 330: 349-372, 1982.

Auquet, M. and DeFeudis, F.V.: Actions of D600 on isolated rabbit aorta. Gen. Pharmac.. 13: 343-346, 1983.

Awad, R., Payne, R. and Deth, R.C.: Alpha adrenergic receptor subtype associated with receptor binding, Ca++ influx, Ca++ release and contractile events in the rabbit aorta. J. Pharmacol. Exp. Ther.. 227: 60-67, 1983.

Barlow, R.B., Scott, N.C. and Stephenson, R.P.: The affinity and efficacy of onium salts on the frog rectus abdominus. Br. J. Pharmacol.. 31: 188-196, 1967.

Barrett, E.F. and Barrett, J.N.: Separation of two voltage - sensitive potassium currents, and demonstration of a tetrodotoxin - resistant calcium current in frog motorneurons. J. Physiol.. 255: 737-774, 1976.

Berridge, M.J.: Phosphatidylinositol metabolism and calcium gating in a 5-HT receptor system, In: Drug Receptors and their Effectors, ed. by N.J.M. Birdsall. Biological Council, The Co-ordinating Committee for Symposia on Drug Action. 75-85, 1981.

Besse, J.C. and Furchgott, R.F.: Dissociation constants and relative efficacies of agonists acting on alpha adrenergic receptors in rabbit aorta. J. Pharmacol. Exp. Therap.. 197: 66-78, 1976.

Bevan, J.A. and Verity, M.A.: Sympathetic nerve-free vascular muscle. J. Pharmacol. Exp. Ther.. 157: 117-124, 1967.

Bieger, D., Kruger - Thiemer, E., Lullman, H. and Ziegler, A.: The rate of action of cholinergic drugs in guinea - pig isolated atria. Eur. J. Pharmacol.. 9: 156-162, 1970.

Billah, M.M. and Michell, R.H.: Phosphatidylinositol metabolism in rat hepatocytes stimulated by glycogenolytic hormones. Biochem. J.. 182: 661-668, 1979.

Birnbaumer, L., Swartz, T.L., Abramowitz, J., Mintz, P.W. and Iyengar, R.: Transient and steady state kinetics of the interaction of guanyl nucleotides with the adenylyl cyclase system from rat liver plasma membrane. J. Biol. Chem.. 255: 3542-3551, 1980.

Black, J., Gerskowitch, V.P. and Leff, P.: Reflection on the classification of histamine receptors. In: Pharmacology of Histamine Receptors, ed. by C.R. Ganellin and M.E. Parsons. Wright. PSG, UK. 1-9, 1982.

Black, J.W. and Leff, P.: Operational models of pharmacological agonism. Proc. R. Soc. Lond.. B 220: 141-162, 1983.

Boeynaems, J.M. and Dumont, J.E.: Quantitative analysis of the binding of ligands to their receptors. J. Cyclic Nucleotide Res.. 1: 123-142, 1975.

Bohr, D.: Vascular smooth muscle: Dual effect of calcium. Science (Wash. D.C). 139: 579-580, 1963.

Bolton, T.R.: Mechanisms of action of transmitters and other substances on smooth muscle. Physiol. Rev.. 59: 606-718, 1979.

Brehm, P., Eckert, R. and Tillotson, D.: Calcium - mediated inactivation of calcium current in Paramecium. J. Physiol.. 306: 193-203, 1980.

Brodie, D.C., Bohr, D.F. and Smit J: Dual contractile response of the aorta strip. Am, J. Physiol.. 197: 241-246, 1959.

Caroni, P. and Carafoli, E.: The Ca++ - pumping ATPase of Heart Sarcolemma. J. Biol. Chem.. 256: 3263-3270, 1981.

Cassidy, P., Hoar, P.E. and Kerrick, W.G.L.: Irreversible thiophosphorylation and activation of tension in functionally skinned rabbit ileum strips by [35S] - ATP S. J. Biol. Chem.. 254: 11148-11153, 1979.

Cassidy, P.S., Kerrick, W.G.L., Hoar, P.E. and Malencik, D.A.: Exogenous calmodulin increases Ca++ sensitivity of isometric tension activation and myosin phosphorylation in skinned smooth muscle. Plugers Arch.. 392: 115-120, 1981.

Cavero, I. and Spedding, M.: Calcium antagonists: A class of drugs with a bright future. Part I. Cellular calcium homeostasis and calcium as a coupling messenger. Life Sci.. 33: 2571-2581, 1983.

Chang, K.J. and Triggle, D.J.: Quantitative aspects of drug - receptor interactions I. Ca++ and cholinergic receptor activation in smooth muscle : a basic model for drug - receptor interaction. J. Theor. Biol.. 40: 125-154, 1973.

Clancy, B., Silva, W. and Maayani, S.: 5-HT receptors in isolated rabbit aorta: Characterization by a series of 5-HT agonists. Abstract, Federation Proc.. 1983.

Clancy, B.M. and Maayani, S.: The 5-HT receptor in the isolated rabbit aorta: Characterization by tryptamine analogs. In preparation (personal communication). 1984.

Clark, A.J.: The Mode of Action of Drugs on Cells. E. Arnold & Co., London. 1933.

Conn, P.J. and Sanders-Bush, E.: Selective 5-HT2 antagonists inhibit serotonin stimulated phosphatidylinositol metabolism in cerebral cortex. Neuropharmacology. 23: 993-996, 1984.

Cory, R.N., Osman, R. and Maayani, S.: Kinetic characterization of the rabbit aorta contractile response to an alpha adrenergic agonist. J. Pharmacol. Exp. Ther.. 230: 162-170, 1984.

Cuatrecasas, P.: Membrane receptors. Ann. Rev. Biochem.. 43: 169-214, 1974a.

Cuatrecasas, P.: Insulin receptors, cell membranes and hormone action. Biochem. Pharmacol.. 23: 2353-2361, 1974b.

Daniel, E.E., Crankshaw, D.J. and Kwan, C.: Intracellular sources of calcium for activation of smooth muscle. In: Trends in Autonomic Pharmacology, ed. by S. Kalsner. Urban & Schwarzenberg, Baltimore - Munich. 1: 443-484, 1979.

De Lean, A., Hancock, A.A. and Lefkowitz, R.J.: Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for a mixture of phamacological receptor subtypes. Mol. Pharmacol.. 21: 5-16, 1982.

De Lean, A., Stadel, J.M. and Lefkowitz, R.J.: A ternary complex model explains the agonist - specific binding properties of the adenylate cyclase - coupled beta - adrenergic receptor. J. Biol. Chem.. 255: 7108-7117, 1980.

Demaille, J.G. and Pechere, J.F.: The control of contractility by protein phosphorylation, In: Adv. Cycl. Nuc. Res., ed. by P. Greengard and G.A. Robinson. Raven Press, N.Y., N.Y.. 15: 337-371, 1983.

Deth, R. and van Breemen, C.: Inhibition of alpha - receptor induced Ca++ release and Ca++ influx by Mn++ and La+++. Eur. J. Pharmacol.. 71: 1-11, 1981.

Deth, R. and van Breemen, C.: Agonist induced release of intracellular Ca++ in the rabbit aorta. Membrane Biol.. 30: 363-380, 1977.

Deth, R. and van Breemen, C.: Relative contributions of Ca++ release during drug induced activation of the rabbit aorta. Pflugers Arch.. 348: 13-22, 1974.

Deth, R.C. and Casteels, R.: A study of releasable Ca fractions in smooth muscle cells of the rabbit aorta. J. Gen. Physiol.. 69: 401-416, 1977.

Docherty, J.R., Constantine, J.W. and Starke, K.: Smooth muscle of rabbit aorta contains alphal but not alpha2 - adrenoreceptors. Naunyn Schmiedeberg´s Arch. Pharmacol.. 317: 5-7, 1981.

Docherty, J.R. and Starke, K.: Postsynaptic alpha - adrenoreceptor subtypes in rabbit blood vessels and rat anococcygeus muscle studies in vitro. J. Cardiovasc. Pharmacol.. 3: 854-866, 1981.

Eckert, R. and Tillotson, D.L.: Calcium - mediated inactivation of the calcium conductance in caesium - loaded giant neurones of Aplysia Californica. J. Physiol.. 314: 265-280, 1981.

Furchgott, R.F.: The pharmacology of vascular smooth muscle. Pharmacol. Rev.. 7: 183-265, 1955.

Furchgott, R.F.: The use of beta - haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor agonist complexes, In: Advances in Drug Research, ed. by N.J. Harper and A.B. Simmonds. Academic Press, London. vol. 3: 1966.

Furchgott, R.F.: Receptor mechanisms. Ann. Rev. Pharmacol.. 4: 21-50, 1964.

Furchgott, R.F. and Bursztyn, P.: Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. Ann. N.Y. Acad. Sci.. 144: 882-889, 1967.

Gaddum, J.H. and Picarelli, Z.P.: Two kinds of tryptamine receptors. Br. J. Pharmacol.. 12: 323-328, 1957.

Gershengorn, M.C. and Thaw, C.: Calcium influx is not required for TRH to elevate free cytoplasmic calcium in GH3 cells. Endocrinology. 113: 1522-1524, 1983.

Gettes, L.S.: Possible role of ionic changes in the appearance of arrythmias. Pharmac. Ther.. B 2: 787-810, 1976.

Glossmann, H., Ferry, D.R., Lubbecke, F., Mewes, R. and Hofmann, F.: Calcium channels: direct identification with radioligand binding studies. Trends Pharmacol. Sci. 3: 431-437, 1982.

Godfraind, T. and Kaba, A.: Actions phasique et tonique de l´adrenaline sur un muscle lisse vasculaire et leur inhibition par des agents pharmacologiques. Arch. Int. Pharmacodyn.. 178: 488-491, 1969.

Gordon, A.R.: Contraction of detergent - treated smooth muscle. Proc. Natl. Acad. Sci.. 75: 3527-3530, 1978.

Hartshorne, D.J.: The contractile apparatus of smooth muscle and its regulation by calcium, In: Vascular Smooth Muscle: Metabolic, Ionic and Contractile Mechanisms, ed. by M.F. Crass and C.D. Barnes. Academic Press, N.Y., N.Y.. 135-161, 1982.

Hartshorne, D.J. and Gorecka, A.: Biochemistry of the contractile proteins of smooth muscle, In: Handbook of Physiology, ed. by D.F. Bohr, A.P. Somlyo, and H.V. Sparks. Am. Physiol. Soc., Bethesda, Md.. section 2, vol. 2: 93-120, 1980.

Hester, R.K., Weiss, G.B. and Fry, W.J.: Differing actions of nitroprusside and D-600 on tension and 45Ca fluxes in canine renal arteries. J. Pharmacol. Exp. Ther.. 208: 155-160, 1979.

Hoar, P.E., Kerrick, W.G.L. and Cassidy, P.S.: Chicken gizzard: relation between calcium - activated phosphorylation and contraction. Science. 204: 503-506, 1979.

Hudgins, P.M.: Some drug effects on calcium movements in aortic strips. J. Pharmacol. Exp. Ther.. 170: 303-310, 1969.

Humphrey, P.P.A., Feniuk, W. and Watts, A.D.: Ketanserin - a novel hypertensive drug?. J. Pharm. Pharmac.. 34: 541, 1982.

Isenberg, G.: Cardiac Purkinje fibers: Resting, action, and pacemaker potential under the influence of Ca++ as modified by intracellular injection techniques. Pflugers Arch.. 371: 77-85, 1977.

Jacobs, S. and Cuatrecasas, P.: The mobile receptor hypothesis and "cooperativity" of hormone binding. Application to insulin. Biochim. Biophys. Acta. 433: 482-495, 1976.

Jung, A., Lullmann, H. and Ziegler, A.: An attempt to correlate kinetic data of drug - receptor interaction with the chemical structures of cholinomimetic agents. Eur. J. Pharmacol.. 15: 327-334, 1971.

Karaki, H., Kubota, H. and Urakawa, N.: Mobilization of stored calcium for phasic contraction induced by norepinephrine in rabbit aorta. Eur. J. Pharmacol.. 56: 237-245, 1979.

Karaki, H. and Weiss, G.B.: Alterations in high and low affinity binding of 45Ca in rabbit aortic smooth muscle by norepinephrine and potassium after exposure to lanthanum at low temperature. J. Pharmacol. Exp. Ther.. 211: 86-92, 1979.

Katz, A.M., Messineo, F.C. and Herbette, L.: Ion channels in membranes. Circulation. 65 (Suppl. I): I 2-10, 1982.

Keene, J.J., Seidel, C.L. and Bohr, D.F.: Effect of manganese on calcium flux and norepinephrine - induced tension in arterial smooth muscle. Proc. Soc. Exp. Biol. Med.. 139: 1083-1085, 1979.

Kenakin, T.P. and Black, J.W.: The pharmacological classification of practolol and choropractolol. Mol. Pharmacol.. 141: 607-623, 1978.

Kerrick, W.G.L. and Hoar, P.E.: Inhibition of smooth muscle tension by cyclic AMP - dependent protein kinase. Nature. 292: 253-255, 1981.

Kerrick, W.G.L., Hoar, P.E. and Cassidy, P.S.: Calcium - activated tension: the role of myosin light chain phosphorylation. Federation Proc.. 39: 1558-1563, 1980.

Kirk, C.J., Michell, R.H. and Hems, D.A.: Phosphatidylinositol metabolism in rat hepatocytes stimulated by vasopressin. Biochem. J.. 194: 155-165, 1981.

Lass, Y., Akselrod, S., Gavish, B., Cohen, S. and Fisher, A.: Offset of rate of action of muscarinic anatgonists depends on their structural flexibility. Experientia. 35: 650-652, 1979.

Leysen, J.E., Niemegeers, C.J.E., Van Nueten, J.M. and Laduron, P.M.: 3H-Ketanserin (R41 648), a selective 3H-ligand for serotonin2 receptor binding sites. Mol. Pharmacol.. 21: 301-314, 1982.

Loutzenhiser, R. and van Breemen, C.: The influence of receptor occupation on Ca++ influx mediated vascular smooth muscle contraction. Circ. Res.. 52 (Suppl. I): 97-103, 1983.

Maayani, S., Wilkinson, W.C. and Stollak, J.: 5-HT receptor in the rabbit aorta: characterization with butyrophenone analogs. J. Pharmacol. Exp. Ther.. 229: 346-350, 1984.

Maxwell, R.A., Eckhardt, S.B. and Wastila, W.B.: Concerning the distribution of endogenous norepinephrine in the advential and media - intimal layers of the rabbit aorta and the capacity of these layers to bind tritiated norepinephrine. J. Pharmacol. Exp. Ther. . 161: 34-39, 1968.

Meisheri, K., Hwang, O. and van Breemen, C.: Evidence for two separate Ca++ pathways in smooth muscle plasmalemma. J. Membane Biol.. 59: 19-25, 1981.

Meisheri, K.D. and van Breemen, C.: Effects of beta - adrenergic stimulation on calcium movements in rabbit aortic smooth muscle: relationship with cAMP. J. Physiol. (Lond.). 331: 429-441, 1982.

Metzler, D.E.: Biochemistry. Academic Press, New York. 198, 1977.

Michell, R.H.: Inositol phospholipids and cell surface receptor function. Biochim. Biophys. Acta. 415: 81-147, 1975.

Michell, R.H., Kirk, C.J., Jones, L.M., Downes, C.P. and Creba, J.A.: The stimulation of inositol lipid metabolism that accompanies calcium mobilization in stimulated cells: defined characteristics and unanswered questions. Phil. Trans. Roy. Soc. Lond.. B 296: 123-137, 1981.

Mrwa, U. and Hartshorne, D.J.: Phosphorylation of smooth muscle myosin and myosin light chains. Federation Proc.. 39: 1564-1568, 1980.

Mueller, E. and van Breemen, C.: Role of intracellular Ca++ sequestration in beta - adrenergic relaxation of smooth muscle. Nature. 281: 682-683, 1979.

Mukai, T. and Kubota, K.: Differences in contractile responses to acetylcholine and serotonin in rat stomach fundus strips. Eur. J. Pharmacol.. 65: 157-163, 1980.

Murphy, K.M.M., Gould, R., Largent, B.L. and Snyder, S.H.: A unitary mechanism of calcium antagonist drug action. Proc. Natl. Acad. (USA). 80: 860-864, 1983.

Nelson, T.E. and Tooke, R.M.: Biphasic adrenergic responses of rabbit aorta. Microvasc. Res.. 8: 172-180, 1974.

Paton, W.D.M.: A theory of drug action based on the rate of drug - receptor combination. Proc. Royal Soc. London. 154: 21-69, 1961.

Peroutka, S.J. and Snyder, S.H.: Multiple serotonin receptors: Differential binding of [3H]-5-hydroxytryptamine, [3H]-lysergic acid diethylamide and [3H]-spiroperidol. Mol. Pharmacol.. 16: 687-699, 1979.

Putney, J.W., Weiss, S.J., Van De Walle, C.M. and Haddas, R.: Is phosphatidic acid a calcium ionophore under neurohumoral control?. Nature. 284: 345-347, 1980.

Ratz, P.H. and Flaim, S.F.: Mechanism of 5-HT contraction in isolated bovine ventricular coronary arteries. Circ. Res.. 54: 135-143, 1984.

Reuter, H.: Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature. 301: 569-574, 1983.

Ruffolo, R.R.: Important concepts of receptor theory. J. Auton. Pharmac.. 2: 277-295, 1982.

Saida, K. and van Breemen, C.: Inhibiting effect of diltiazem on intracellular Ca++ release in vascular smooth muscle. Blood Vessels. 20: 105-108, 1983.

Salaices, M., Marin, J., Rico, M.L. and Gonzales, C.: Effects of verapamil and manganese on the vasoconstriction responses to noradrenaline, serotonin, and potassium in human and goat cerebral arteries. Biochem. Pharmacol.. 32: 2711-2714, 1983.

Salmon, D.M. and Honeyman, T.W.: Proposed mechanism of cholinergic action in smooth muscle. Nature. 284: 344-345, 1980.

Schaub, M.C., Watterson, J.G. and Waser, P.G.: Pharmacology and muscle contraction. Trends Pharmacol. Sci.. 4: 116-120, 1983.

Schild, H.O.: The use of drug antagonists for the identification and classification of receptors. Br. J. Pharmacol.. 2: 251-258, 1947.

Schulman, H.: Calcium - dependent protein phosphorylation, In: Handbook of Experimental Pharmacology, ed. by G.V.R. Born, A. Farah, H. Herken, and A.D. Welch. Springer Verlag, Berlin. 58, section 2: 425-463, 1982.

Sheys, E.M. and Green, R.D.: A quantitative study of alpha adrenergic receptors in the spleen and aorta of the rabbit. J. Pharmacol. Exp. Therap.. 180: 317-325, 1972.

Sparrow, M.P., Mrwa, U., Hofmann, F. and Ruegg, J.C.: Calmodulin is essential for smooth muscle contraction. FEBS Letters. 125: 141-145, 1981.

Standen, N.B.: Ca channel inactivation by intracellular Ca injection into Helix neurones. Nature. 293: 158-159, 1981.

Steinsland, O.S., Furchgott, R.F. and Kirkpekar, S.M.: Biphasic vasoconstriction of the rabbit ear artery. Circ. Res.. 32: 49-58, 1973.

Stephenson, R.P.: A modification of receptor theory. Br. J. Pharmacol.. 11: 379-393, 1956.

Stollak, J.: Pharmacological characterization of the receptors for 5-hydroxytryptamine and tryptamine in the rabbit thoracic aorta. THESIS - Downstate Medical Center, Brooklyn, N.Y.. 1980.

Stollak, J.S. and Furchgott, R.F.: Use of selective antagonists for determining the types of receptors mediating the action of 5-hydroxytryptamine and tryptamine in the isolated rabbit aorta. J. Pharmacol. Exp. Ther.. 224: 215-221, 1983.

Sunano, S. and Miyazaki, E.: The initiation of contraction by extracellular calcium in smooth muscle of the guinea - pig taenia coli. Experientia. 24: 364-365, 1968.

Talitman, T.: On the Transition of Agonistic into Antagonistic Muscarinic Activity. M. Sc. Thesis. Tel - Aviv University: 1976.

Tillotson, D.: Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. Proc. Natl. Acad. Sci. (USA). 76: 1497-1500, 1979.

Tolkovsky, A.M.: The elucidation of some aspects of receptor function by the use of a kinetic approach. Current Topics in Membranes and Transport. 18: 11-44, 1983.

Tolkovsky, A.M., Braun, S. and Levitzki, A.: The kinetics of interaction between beta receptors, the GTP protein and the catalytic unit of turkey erythrocyte adenylate cyclase. Proc. Natl. Acad. Sci. (U.S.A.). 79: 213-217, 1982.

Tolkovsky, A.M. and Levitzki, A.: Mode of coupling between the beta - adrenergic receptor and adenylate cyclase in turkey erthrocytes. Biochemistry. 17: 3795-3810, 1978.

Tolkovsky, A.M. and Levitzki, A.: Theories and predictions of models describing sequential interactions between the receptor, the GTP regulatory subunit and the catalytic unit of hormone dependent adenylate cyclases. J. Cyclic Nucleotide Res.. 7: 139-150, 1981.

Triggle, D.J. and Chang, K.J.: Quantitative aspects of drug - receptor interactions. I. Ca++ and cholinergic receptor activation in smooth muscle: a basic model for drug - receptor interactions. J. Theor. Biol.. 40: 125-154, 1973.

Tsai, B.S. and Lefkowitz, R.J.: [3H]-Dihydroergocryptine binding to alpha adrenergic receptors in canine aortic membranes. J. Pharmacol. Exp. Ther.. 204: 606-614, 1978.

Tsien, R.Y.: New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry. 19: 2396-2404, 1980.

Tsien, W.: Calcium channels in excitable cell membranes. Ann. Rev. Physiol.. 45: 341-358, 1983.

Tyson, C.A., Vande Zande, H. and Green, D.E.: Phospholipids as ionophores. J. Biol. Chem.. 251: 1326-1332, 1976.

van Breemen, C.: Blockade of membrane calcium fluxes by lanthanum in relation to vascular smooth muscle contractility. Arch. Int. Physiol. Biochim.. 77: 710-716, 1969.

van Breemen, C., Aaronson, R., Loutzenhiser, R. and Meisheri, K.: Calcium fluxes in isolated rabbit aorta and guinea pig tenia coli. Fed. Proc.. 41: 2891-2897, 1982.

van Breemen, C., Farinas, B.R., Gerba, P. and McNaughton, E.D.: Excitation - contraction coupling in rabbit aorta studied by the lanthanum method for measuring cellular calcium influx. Circ. Res.. 30: 44-54, 1972.

van Breemen, C., Hwang, O. and Meisheri, K.D.: The mechanism of inhibitory action of diltiazem on vascular smooth muscle contractility. J. Pharmacol. Exp. Ther.. 218: 459-463, 1981.

van Ginnekin, C.A.M.: Kinetics of drug - receptor interaction, In: Kinetics of Drug Action, the Handbook of Experimental Pharmacology, ed. by J. M. van Rossum. Springer - Verlag, Berlin. vol. 47: 357-411, 1977.

Waud, D.R.: Pharmacological receptors. Pharmacol. Rev.. 20: 49-88, 1968.

Webb, C.R.: D-600 and the membrane stabilizing effect of calcium in vascular smooth muscle. Pharmacology. 25: 250-261, 1982.

Wurzel, M.: Serotonin receptor in rabbit artery. Am. J. Physiol.. 211: 1424-1428, 1966.