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Functional interactions in smooth muscle

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FUNCTIONAL INTERACTIONS IN SMOOTH MUSCLE

BY

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Abstract

Functional Interactions in Smooth Muscle

by

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Vascular smooth muscle tone is modulated *in vivo* by the functional interaction of a variety of vasoconstrictor and vasodilator stimuli. Endogenous substances (e.g. epinephrine) simultaneously activate α-adrenergic receptors (α-AR) eliciting contraction and ß-adrenergic receptors (ß-AR) which relax the muscle. This study characterizes the ß-adrenergic response in the isolated rabbit aorta precontracted with phenylephrine (PE) or 5HT. The ß-adrenergic agonist isoproterenol (ISO) produces a biphasic response that is composed of a rapid relaxation followed by a slower regaining of tension which is identified as desensitization. An exploratory kinetic model that describes both relaxation and desensitization (as simple exponential functions) provides a good fit to the experimental data. The five parameters used to describe the ISO response are: the observed rate constants for

relaxation and desensitization (k_{rel} and k_{des}), the magnitudes of the changes in tension for the two processes (**R** and **D**), and the observed delay in the onset of desensitization response, t_d. The k_{rel} and fractional relaxation were dependent on concentration of ISO in a saturable manner in rings precontracted with 1 μM PE (EC50 = 0.017 μM and 0.067 μM , respectively). No concentration dependences were observed for k_{des}, fractional desensitization **(D/R)** and t_d (average values ± SEM are $(4.7 \pm 0.2)*10^{-3}$ sec^{-1}; 0.83 ± 0.02; 191 ± 6 sec, respectively). This work demonstrates that a kinetic analysis is necessary to properly estimate the parameters that describe the relaxation response to ISO when relaxation is accompanied by simultaneous desensitization.

The ß-adrenergic response parameters were also characterized in aortic rings precontracted with various concentrations of PE or 5HT and under conditions that varied the efficacy of the contractile agonist. The k_{rel} and fractional relaxation were both inversely related to the concentration of PE. The k_{rel} was also inversely related to the concentration of 5HT, however, the fractional relaxation changed little with 5HT concentration (**R / C** ~ 0.8). Although fractional desensitization **(D/R)** showed no dependence, k_{des} and t_d were dependent on the concentration of PE or 5HT in a saturable manner and also varyed with the efficacy of the contractile

agonist. k_{des} increases with increasing concentration of PE or 5HT (EC50= 0.43 μM or 0.08 μM in the presence of PE and 5HT respectively) and with increasing efficacy whereas t_d decreases under these same conditions. The lack of dependence of k_{des} and t_d on the concentration of ISO and the dependence of these parameters on the concentration of PE or 5HT and on contractile efficacy suggest that there is a relationship between events that mediate contraction and ß-AR desensitization.

Acknowledgements

I must begin by thanking Drs. Melman and Maayani who four years ago mentioned to me that there was an "MD/PhD" program here at Mount Sinai. Although I knew that some few individuals might be interested in such a program, I learned during my short summer with Dr. Melman that I was one of them. Having made it to this point, I must acknowledge those who have contributed their love and support throughout my endless studies and during the preparation of this manuscript: my husband Paul, my babysitter Joycelyn, my 2 year old son Adam (who was always asking his daddy when the "sheri-monster" was coming home from work) and I must not leave out my beloved Mac.

I will not thank my advisors, Rami, Joe and Saul, in this document for their guidance and support. Rather, I will defer these thanks until a time when I will repay them by being equally dedicated to my own students. I very much hope that as an advisor and friend I will be able to change the course of even one of my students lives to the extent that my advisors have changed mine. I will thank Rami for sharing his family with me, especially Miriam who has always been warm, loving and a joy.

Finally, I dedicate this work, as I do all my work, to the memory of my mother who gave me all I have to give.

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Abbreviations

AC	adenylyl cyclase
α-AR	a-adrenergic receptor
ß-AR	ß-adrenergic receptor
ß-ARK	ß-adrenergic receptor kinase
Bay K	Bay K 8644
BUF	Bufotenin
cAMP	cyclic AMP
CRC	concentration response curve
DHP	dihydropyridine
DMT	N,N dimethyl tryptamine
D•R	drug-receptor complex
G_s	stimulatory GTP-binding protein
HA	histamine
ISO	isoproterenol
NIF	nifedipine
PE	phenylephrine
PKC	protein kinase C
PKA	protein kinase A
PMA	phorbol 12-myristate 13-acetate
QUIP	quipazine
5HT	serotonin

INTRODUCTION.

Few receptors have been more extensively studied than the ß-adrenergic receptor (ß-AR). ß-ARs are present in a variety of biological systems where they mediate a diverse range of physiologic responses. Furthermore, the ß-AR is a prototype for studying hormonal activation of adenylyl cyclase (AC), receptor:GTP-binding protein (G-protein) interactions and receptor desensitization. Over the past two decades, the molecular events that lead to the activation of the ß-AR-linked AC and the attenuation of that activation, have contributed greatly to the understanding of signalling systems that employ G proteins as transducing elements. Furthermore, ß-ARs mediate important physiologic processes such as control of smooth muscle tone, heart rate and cardiac contractility and both ß-AR agonists and antagonists are important therapeutically.

The molecular events leading to activation of AC, the role of GTP-binding proteins: The hormonally regulated AC is composed of three separate components: the membrane receptor (e.g. the ß-AR), the stimulatory G protein (G_s) and the catalytic unit (C) of the adenylyl cyclase enzyme. The stimulatory GTP binding protein is a heterotrimer: the stimulatory α-subunit (α_s), and the ß and γ subunits. The α_s contains a guanine nucleotide binding site. In the inactive

form of G_s, GDP is bound to α_s. Activation occurs upon dissociation of GDP and subsequent binding of GTP to α_s. It is the role of the activated membrane receptor to facilitate this change, acting very much like a catalyst to speed up the otherwise slow dissociation of GDP. GTP binding promotes the dissociation of the α-subunit of G_s from the $\beta\cdot\gamma$ complex which remains embedded in the membrane. The β and γ subunits are tightly associated and can only be separated under denaturing conditions. The β and γ subunits are thought to inhibit the actions of α_s when the heterotrimer is intact and also to anchor the protein in the membrane. The dissociation of the heterotrimer appears to be the rate limiting step in the process of AC activation and allows α_s to activate the catalytic subunit. Inactivation of α_s is brought about by the hydrolysis of GTP to GDP and the reassociation of the heterotrimeric $\alpha\beta\gamma$ subunit (reviewed in Gilman, 1986; Levitzki, 1987; Iyengar and Birnbaumer, 1987; Weiss et al., 1988).

The kinetic features of AC activation, the collision-coupling model: A kinetic study of the activation of AC by the β-AR in turkey erythrocytes was done by Tolkovsky and Levitzki (1978,1981; Tolkovsky et al., 1982). By focusing on the temporal aspect of cAMP accumulation, they demonstrated that there was a lag time prior to the accumulation of cAMP

and that this lag time could be decreased up to a limiting value by increasing the concentration of ß-adrenergic agonist. Thus, the lag time was dependent on the concentration of agonist in a saturable manner. The authors demonstrated that this behavior was consistent with a kinetic model in which the slow activation of an effector by the drug•receptor complex (D•R) was the rate-limiting step.

Tolkovsky and Levitzki derived an observed rate constant for this process by integrating the curves that described the cAMP accumulation. This observed rate constant was dependent on the concentration of agonist in a saturable manner and displayed a linear dependence on the total number of receptors which was demonstrated by using an irreversible ß-adrenergic antagonist). Because a rate constant always reflects the rate-limiting step of a process, the slowest step in the process of cAMP formation was postulated to be the activation of the effector by D•R.

The isolation of specific components of the AC-linked ß-AR allowed this model to be described explicitly. The effector can be explicitly defined as the G_S•C complex. The activated receptor, acting as a catalyst for the activation of the G_S•C, only briefly forms a loose complex with G_S•C and therefore may activate more than one G_S•C complex. The G_S•C activation is a first order process and the rate constant of this process

depends linearly on the total number of receptors (Levitzki, 1987). This catalytic process has been termed the 'collision coupling' model; it identifies the rate limiting step of the accumulation of cAMP as activation of the G_S.

Attenuation of AC activation, desensitization of the ß-AR: Attenuation of responsiveness in the continued presence of agonist is a widely recognized form of regulation of signal transduction pathways. The decrease in responsiveness of the ß-AR has served as the primary model for the study of receptor desensitization. Sibley et. al (1984) suggested that receptor phosphorylation is correlated with a decrease in adenylyl cyclase activity in turkey erythrocytes and began the continuing search for the identity of the kinase(s) responsible for desensitization *in vivo*. Subsequently the ß-AR has been shown to be phosphorylated by at least 3 distinct kinases: ß-adrenergic receptor kinase (ß-ARK; Benovic et al., 1986), cAMP-dependent protein kinase (PKA; Benovic et al., 1985; Bouvier et al., 1987), and protein kinase C (PKC; Bouvier et al., 1987). There is still considerable debate concerning which kinase(s) mediate ß-adrenergic desensitization.

ß-ARK is a cAMP independent kinase that specifically phosphorylates the agonist-occupied ß-AR as well as several other receptors coupled to adenylyl cyclase (Benovic et al., 1986). ß-ARK has been hypothesized to phosphorylate the ß-

AR and thereby mediate homologous desensitization. In one scheme that is proposed to explain ß-AR desensitization, homologous (receptor specific) desensitization of the ß-AR is thought to be initiated by the translocation of ß-ARK from the cytosol to the plasma membrane (Strasser et al., 1986b). Once translocated to the membrane, ß-ARK appears to phosphorylate the activated form of the receptor, i.e., the receptor occupied by an agonist (Benovic, 1986). The phosphorylated receptor is functionally uncoupled from G_s and thereby incapable of activating the adenylyl cyclase system. Subsequently, the phosphorylated receptors are sequestered into the cell cytosol. The internalized receptors are dephosphorylated and can be recycled back into the membrane. With long exposure times to agonist, the receptors undergo degradation (reviewed in Benovic, 1988; Fishman and Perkins, 1988; Sibley and Lefkowitz, 1987).

Several attempts have been made to establish the importance of ß-ARK in homologous desensitization. Bouvier et al. (1988) showed that the removal of possible ß-ARK phosphorylation sites from the $ß_2$AR delayed but did not abolish desensitization (defined as loss of AC activity). Lohse et al. (1989) used recently described inhibitors of ß-ARK in permeabilized human epidermoid carcinoma A431 cells to inhibit ß-AR phosphorylation and desensitization.

Furthermore, inhibitors of cAMP-dependent protein kinase and protein kinase C (e.g. H7) were not effective inhibitors of homologous desensitization in these permeabilized cells (Lohse et al. 1989). These findings support the hypothesis that ß-AR desensitization in some systems may be largely mediated through the actions of ß-ARK and not via PKA or PKC.

In contrast, other studies suggest important roles for PKA and PKC in agonist-induced ß-AR regulation. Investigators have implicated PKA in heterologous desensitization of adenylyl cyclase in S49 lymphoma cells (Sibley et al., 1984; Clark et al., 1988) and PKC has been shown in many studies to decrease ß-AR induced adenylyl cyclase activity (Yamashita et al., 1987; Toews et al., 1987; Hui and Yu,1989) Toews et al. (1987) showed that the PKC activator, PMA, could lead to a decrease in adenylyl cyclase activity in 1321N1 human astrocytoma cells but that this decrease in adenylyl cyclase activity has different characteristics than desensitization induced by ISO in the same cells. Toews et al. proposed that PMA treatment results in desensitization that is presumably mediated through phosphorylation of the ß-AR but occurs without internalization and which is not specific for a particular receptor whereas ISO induces homologous (receptor specific) phosphorylation and internalization of the ß-AR. Thus it follows that the combination of PMA + ISO would lead

to a greater desensitization than either agent alone in this cell line. Yamashita et al. (1987) confirmed that PMA induced desensitization was enhanced by ISO activation of the ß-AR.

Activation of ß-AR's in a functional system: In smooth muscle preparations, the response to the ß-adrenergic agonist isoproterenol (ISO) is relaxation of precontracted tissue. Van den Brink (1973) described functional antagonism as the inverse relationship between the degree of relaxation induced by ISO (measured as inhibition of contractile response) and the level of contraction elicited by methacholine or histamine in the guinea pig trachea. This inverse relationship has been described by many investigators in tracheal smooth muscle preparations (Van den Brink, 1973; Buckner and Saini, 1975; Torphy et al., 1983). The functional antagonism has been most often characterized by changes in the parameters that describe the steady state concentration response curves for the relaxant effect of ISO (i.e., an increase in EC_{50} value and a decrease in E_{max} as the concentration of muscarinic agonist is increased).

Furthermore, there are numerous reports that the relaxant effect of ISO varies with the identity as well as the concentration of the agonist eliciting the contractile response (Torphy, 1984; Van Amsterdam, 1989). In tracheal smooth muscle, ISO or PGE_2 induced relaxation was consistently least

effective at high concentrations of the muscarinic agonists, acetylcholine and methacholine and was most effective at high concentrations of HA, 5HT and leukotriene D_4 (Russell, 1984; Torphy, 1984; Madison et al., 1989; Van Amsterdam et al., 1989).

The present investigation: ß-adrenergic response in the rabbit aorta preparation: Previous studies of functional antagonism were all performed in tracheal smooth muscle where the relaxation response to ISO is stable for up to 30 minutes (unpublished observation). However, in the rabbit aorta preparation, desensitization of the ß-AR can be visualized as a rapid loss of ISO induced relaxation response. Thus, the rabbit aorta preparation presents a unique opportunity to study both the functional response to ISO (relaxation) and the loss of this response (desensitization) in an intact tissue preparation where functional interactions between activated receptor:effector systems can be observed.

I report here on my investigations on the kinetics of the ISO induced relaxation and desensitization in the isolated rabbit aorta. A heuristic kinetic model was developed in order to separate these two responses. The parameters of the model are the steady state magnitudes of relaxation and desensitization as well as three kinetic parameters: the observed rate constant for the relaxation response, the

observed rate constant for the desensitization response and a delay or lag time that occurs following ISO addition before the onset of desensitization. The parameters were characterized with respect to several factors: the concentration of the ß-adrenergic agonist ISO, the concentration of agonist eliciting the contraction (either phenylephrine or 5HT), and the efficacy of the agonist eliciting contraction. Specifically, the dependencies of the kinetic parameters of the model on the concentration of agonists were used to propose mechanistic hypotheses to explain the observed responses.

paper I

Functional Interactions in Smooth Muscle: Kinetic Characterization of the Relaxation and Desensitization Responses to a ß-adrenergic Agonist in the Rabbit Aorta.

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Vascular smooth muscle tone is regulated *in vivo* by time dependent interactions between stimuli that mediate vasoconstriction and vasodilation. These opposing responses can be studied using an intact smooth muscle preparation such as the isolated rabbit aorta. The goal of this work was to quantitate the functional antagonism between a vasoconstrictor, phenylephrine (PE) and a vasodilator, isoproterenol (ISO). The simultaneous desensitization of the ß-adrenergic receptor (ß-AR) makes the quantitation of this functional antagonism impossible using only steady state measurements. Therefore, it was necessary to apply a kinetic approach to study the functional antagonism.

The functional response to ß-AR activation in vascular smooth muscle is relaxation of a precontracted tissue. Furchgott and Bhadrakom (1953) and others (Dorevitch, 1968; Fleisch et al, 1970, Fleisch and Titus 1972; Fleisch and Hooker, 1976; Turlapaty, 1975) described the relaxation response to ISO in the rabbit aorta precontracted with a variety of agonists, acetylcholine, histamine, serotonin, norepinephrine or epinephrine. Furchgott (1953) and Fleisch et al. (1970) noted, however, that the CRC to ISO was biphasic; at low concentrations the tissue relaxed with each addition of ISO, and at higher concentrations of ISO a contraction was

observed. This loss of relaxation at higher concentrations was attributed to the activation of the α-adrenergic receptor (α-AR) in spite of the fact that it occurred in the presence of the α-AR blocker, phentolamine (Fleisch, 1970). The desensitization response initiated by ISO in smooth muscle was not invoked as the explanation of the observed contraction at higher concentrations of ISO.

Desensitization of the ß-AR has been extensively studied in cell culture where the continued presence of agonist has been shown to induce a homologous (receptor specific) desensitization (reviewed in Benovic et al.,1988; Fishman and Perkins, 1988). Multiple mechanisms have been implicated in the loss of responsiveness to agonists from studies in avian erythrocytes (Stadel et al.,1981), hamster smooth muscle cells (Benovic et al., 1986) or S-49 mouse lymphoma cells (Strasser et al., 1986a). Desensitization was also demonstrated *in vivo* in rat lung following ISO administration (Strasser et. al., 1984) and in vascular smooth muscle following prolonged epinephrine infusion (Tsujimoto and Hoffman, 1984).

Homologous desensitization of the ß-AR may be initiated by the translocation of a kinase called ß-adrenergic receptor kinase (ß-ARK) from the cytosol to the plasma membrane (Benovic 1986, Strasser 1986). Once translocated to the

membrane, ß-ARK appears to phosphorylate the activated form of the receptor, i.e., the receptor occupied by an agonist (Benovic et al., 1986). The phosphorylated receptor is functionally uncoupled from G_s and thereby incapable of activating the adenylyl cyclase system. Subsequently, the phosphorylated receptors are sequestered into the cell cytosol. The internalized receptors are dephosphorylated and can be recycled back into the membrane. With long exposure times to agonist, the receptors undergo degradation (reviewed in Benovic, 1988; Fishman and Perkins, 1988; Sibley and Lefkowitz, 1987). Furthermore, despite the fact that many biological actions mediated by activation of the ß-AR require cAMP, the phosphorylation event is not dependent on cAMP (Strasser et al., 1986a). This work suggests that the relaxation and desensitization processes are elicited via independent pathways following activation of the ß-AR.

In the present work we have explored a kinetic model necessary to quantitate the biphasic response to ISO observed in the rabbit aorta preparation. This approach provides a reliable method for the estimation of parameters that describe the responses to ß-adrenergic agonists in this system and serves as the basis for the formulation of a mechanistic model for the observed functional antagonism and desensitization.

METHODS

Tissue preparation.

Male New Zealand White Rabbits weighing between 1.4-1.8 kg. (Ace Animals, Inc., Boyertown PA) were sacrificed by CO_2 asphyxiation and the thoracic aortae were rapidly excised. The aortae were cleaned by mounting them on a pipet and removing fat and connective tissue. The adventitias were removed by dissection with the aid of a dissecting microscope (Clancy and Maayani, 1985). The adventitia free aortae were stored overnight (12-14 h) at room temperature in Krebs-bicarbonate buffer saturated with 95% O_2-5% CO_2. The tissues were cut into 4-6 rings 0.5-0.6 cm in width which were suspended between stainless steel hooks in 20-ml organ baths. The baths contained Krebs-bicarbonate buffer bubbled with 95% O_2-5% CO_2 to maintain a pH of 7.4 ± 0.2 at 36° ± 1 °C. The tissues were initially set to ~2 g tension and allowed to relax until a stable tension was maintained. The minimum stabilization time was 1 hour. Once resting tension was stabilized, baseline tension was set to a force between 1 and 2 g. All rings were primed twice by eliciting a contraction with 10 μM PE. Drugs were removed from the bath by draining and replacing the buffer.

Solutions.

Krebs-bicarbonate buffer contained the following components (in mM) in glass distilled water: NaCl, 110; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 2.35; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11; Na_2EDTA, 0.03.

Chemicals.

(-)Isoproterenol phosphate, (±)propranolol·HCl; (Sigma, St. Louis, MO); All other chemicals were of analytical grade as previously reported (Clancy and Maayani, 1985).

Sequential responses to ISO.

Figure 1 shows a typical response to ISO. At the end of the desensitization, when tissue tension reached steady state, the tissue was washed three times and left to rest for one hour to allow for recovery of responsiveness. This permitted several sequential assays to be recorded on the same aortic ring (n=50 rings from 13 animals). Sequential concentration response curves (CRC) to ISO were obtained in the presence of 1 μM PE (Fig. 5). The order of testing various concentrations of ISO was randomized.

Estimates of EC_{50}, E_{max} and slope indices (n) were obtained by fitting the logistic equation:

$$E = E_{max}/(1+(EC_{50}/[D])^n) \quad (1)$$

to the means of observed responses (E) as a function of drug concentration [D].

Collection of data points.

Isometric contractions were measured with Grass FT03C force displacement transducers connected to the Grass polygraph model 7D. Signals from the J_6 output of the Grass driver amplifier were fed through a custom made signal conditioner to an 8 channel, 8 bit, analog-to-digital converter controlled with an Apple IIe computer. Data points corresponding to tension were collected at a frequency of 0.04 - 1 Hz. The frequency of sampling was chosen according to the conditions of a particular trial and could be changed at any time during a trial. Each data point is the average of 8 samples collected at a frequency of 666 kiloHz.

The data points were stored on a floppy disk for subsequent analyses and were displayed numerically and graphically on the computer monitor. Fig. 1 shows a representative graph of data points illustrating changes in tissue tension with time in a single aortic ring. Digital values of muscle tension were transferred to a Sun 3/110

workstation for non-linear analyses with the PROPHET II computer program.

The kinetic model:

Figure 1 shows a typical biphasic response to ISO in an aortic ring precontracted with PE. The response to ISO consists of an initial loss of tension (relaxation) which is followed by a regaining of tension (desensitization). We developed a phenomenologic kinetic model which describes the ß-adrenergic response as two temporally distinct processes : relaxation, which begins at the time of administration of ISO and desensitization which is seen after a delay (**td**). As the two processes were separated in time, the relaxation response and the desensitization response were described by two separate equations. The parameters used to describe the ß-adrenergic responses are summarized in the scheme in Fig. 2A.

The relaxation response was described as an exponential decay that begins at the observed peak level of the contractile response (**C**) and reaches a limiting value of **R**(g) at steady state. The relaxation is described by the following equation:

$$T = C - R\,(1 - e^{(-k_{rel} * t)}) \qquad (2)$$

where **T** is the observed tissue tension, **C** is the initial contraction, **R** the ISO induced relaxation, and k_{rel} is the

observed rate constant for relaxation and t is the time elapsed since the addition of ISO. **C** was measured from the experimental curve. The values for **R** and k_{rel} were estimated by fitting experimental data to this model with a non-linear regression procedure.

The desensitization response was also modelled as a simple exponential increase in tension with the addition of a parameter, t_d, which describes the delay time from the addition of ISO to the onset of the desensitization:

$$T = P + D(1- e^{(-k_{des} * (t-t_d))}) \qquad (3)$$

where **T** is the observed tissue tension, **P** is the plateau from which the regaining of tension begins, **D** is the magnitude of the ISO induced desensitization and k_{des} is the observed rate constant for desensitization. The valid domain of equation 3 is only for $t \geq t_d$. The values of **D**, k_{des}, and t_d were estimated using a nonlinear fitting procedure whereas the plateau, **P**, was measured from the tracing.

It can be inferred from Fig. 2 that when t_d approaches zero (i.e. when the desensitization response begins before the relaxation response has reached its maximum) we can no longer accurately measure the value of **P** directly from the tracing. Rather, **P** must be calculated with a time dependent equation. As **P** represents the tension from which desensitization begins, it can be described by eq. 2. Therefore,

when t_d approaches zero, the entire tracing must be described by a single equation in which **P** is replaced by eq. 2 (shown schematically in Fig. 2B):

$$T = C - R\,(1-e^{(-k_{rel} * t)}) + D\,(1-e^{(-k_{des} * t)}) \qquad (3)$$

The values of **R**, k_{rel}, **D**, k_{des}, and t_d were estimated using a nonlinear fitting procedure whereas **C** was measured from the experimental curve. At long times, when the system reaches steady state, the equation reduces to a summation of constants with correct signs (relaxation -negative and desensitization -positive). This yields the final observed level of contraction, **F** (i.e. **F = C - R + D**).

All of the data were evaluated initially with Eq. 2 and 3. However, when the desensitization response begins before the relaxation has reached steady state (i.e. when t_d is less than 5 or 6 times the half-life for relaxation ($T_{1/2}$), Eq. 2 and 3 underestimate all of the parameters that describe the ISO response (Fig. 2B). Therefore, Eq. 3 was used to model experiments in which td $\leq$ 5 times the half-life for relaxation ($T_{1/2} = \ln 2/k_{rel}$). In the fitting, t_d was constrained to be ≥ 0 and R was constrained to be $\leq$ C. In order to compare different rings, the extent of relaxation, is described as fractional relaxation, **R/C**. Likewise, the desensitization is represented as fractional desensitization, **D/R**.

The kinetic model does not include a term to describe the resensitization process. Resensitization can occur since the biphasic response to ISO is completely restored 60 minutes after returning the tissue to resting state by washing of PE and ISO, however, resensitization may not occur in tissue that is activated with PE. If ISO was removed by several washes over a 60 minute period with buffer containing 1 μM PE to maintain the same level of contraction, responsiveness to ISO was <u>not</u> restored even after more than an hour from the first wash (n=12 rings from 3 animals). This suggests that in tissues continuously activated by PE, the desensitization process is not complicated by simultaneous resensitization. This finding is presently under investigation.

RESULTS

ISO induced response in precontracted aorta

The exposure of aortic rings to 1 μM ISO for several minutes produced no measurable effect on basal tissue tone. Therefore, the response to ISO was visualized as the relaxation of precontracted tissue. In these experiments, contraction was evoked by the addition of PE to the organ bath. During the time required to assay the responses to ISO (<30 min.), the contractile response to PE was stable on the same tissue and reproducible over the course of the experiments (8 to 12 hours).

A single concentration of ISO elicited a biphasic response in aortic rings precontracted with PE (Fig. 1). In this series of experiments, quantitative analysis was done by fitting tension data points exclusively to Eq (2) and (3) (i.e. t_d was always ≥ 5 half-lives of relaxation). However, in other work which will be described in subsequent reports Eq.. 4 was used (Methods). Figure 3 shows a representative fit where the relaxation and desensitization appear as 2 temporally distinct portions of the response that are separated by a lag time. In this trial, the first 61 data points were fit to the model that describes the relaxation response (Eq. 2) and the last 41 points were fit to the model that describes the desensitization

response (Eq. 3). The middle 25 points (in the shaded area) were not used. For all fits $r^2>0.96$ and $p< 0.05$ were considered significant.

Concentration dependence of ISO induced relaxation

Due to the simultaneous occurrence of relaxation and desensitization, the relaxation response cannot be accurately quantitated from a cumulative CRC (Fig. 4). Therefore a series of single ISO concentrations was tested sequentially in an aortic ring. Fig. 5 illustrates a representative series of responses to various concentrations of ISO from five consecutive trials. The precontraction produced by 1 μM PE showed minimal variation between trials.

CRC's to ISO were performed in the aortic rings precontracted with a constant concentration of PE (1μM). At this concentration of PE, the values of both parameters that describe the relaxation response, **R/C** and $\mathbf{k_{rel}}$, were dependent on concentration of ISO in a saturable manner (Fig. 6). The parameters were obtained from fitting the means of the data points to the logistic equation (Eq. 1). The parameters are: for **R/C**, EC_{50} = 0.067 μM; $(\mathbf{R/C})^{max}$ = 0.75 (i.e., ISO can relax only 75% of the contraction induced by this concentration of PE); slope index=1.1 and for $\mathbf{k_{rel}}$, EC_{50} = 0.017 μM;

$(k_{rel})^{max}$= 0.044 sec^{-1}; slope index = 1.0 ± 0.2 (n=16 rings from 6 animals). The point at the highest concentration of ISO (10 μM) was omitted in the fit of the points to the logistic equation. At this concentration, it is possible that non-specific effects of ISO (e.g. action on receptors other than ß-AR) may contribute to the deviation of this point from the curve.

Characterization of ISO-induced desensitization

After exposure of the tissue to ISO concentrations ≥1μM, a subsequent exposure of the aorta to ISO had no significant effect on tissue tension (Fig. 7). However, addition of 4 mM $NaNO_2$ elicited a rapid and stable relaxation. Furthermore, agents that raise cyclic–AMP levels, isobutylmethylxanthine (10 μM) and forskolin (10 μM), similarly relaxed a preparation that was unresponsive to ISO (not shown). These data suggest that the loss of responsiveness to ISO is not due to a loss in the tissue's ability to relax, but rather due to desensitization of the response to ISO.

Preincubation with 1 μM (±) propranolol (30 min) blocked the response to 1 μM ISO (n=24 rings from 6 animals). Furthermore, addition of 1 μM propranolol when the ISO induced response reached steady state at the end of the desensitization returned the tissue tension to the level

produced by PE alone prior to the addition of ISO (n=12 rings from 3 animals; not shown).

To show that loss of tissue responsiveness to ISO was not solely due to degradation of ISO or release of a stable endogenous ß-AR antagonist, the buffer solution was collected from an organ bath at the end of an assay and was tested on a fresh tissue. The fresh tissue was precontracted with the same concentration of PE (1 μM) and once contraction stabilized, the buffer was replaced with the collected buffer containing PE (1 μM) and ISO (1 μM). A biphasic response was observed: relaxation followed by loss of the relaxation response (n=4 repetitions from 2 animals). The responses to ISO in the pair of rings were qualitatively similar.

Dependence of desensitization on ISO concentration.

At ISO concentrations below 0.03 μM, the changes in tissue tone were too small to allow accurate or statistically significant estimates of parameters for **D** or $\mathbf{k_{des}}$. The values of the two parameters that describe the desensitization response, **D/R**, and $\mathbf{k_{des}}$, show no dependence on concentration of ISO for concentrations 0.03 μM and above. In this range, the average **D/R** is 0.83 ± 0.02 (n=63 repetitions from 6 animals). In other words, 83% of the relaxation response is desensitized. The average $\mathbf{k_{des}}$ is $(4.7 \pm 0.2)*10^{-3}$ sec^{-1} over a range of ISO concentrations varying from 0.03 μM to 10 μM (n=67

repetitions from 6 animals). The parameter t_d was also not dependent on concentration of ISO. The average t_d is 191 ± 6 seconds for ISO concentrations ranging from 0.01 μM to 10 μM (n=73 repetitions from 6 animals).

DISCUSSION.

In vascular smooth muscle, the relaxation response to ISO can be complicated by the simultaneous occurrence of desensitization. This complication necessitates a kinetic analysis of the observed data for several reasons. First, the addition of ISO to a precontracted aortic ring yields two rapid responses, relaxation and desensitization, that alter tissue tone in opposing directions. When these responses overlap in time, the tissue tension does not reach a steady state until both processes are completely finished. In other words, no point on the curve can be taken as a reliable steady state relaxation response. Thus, because the relaxation response to a ß-adrenergic agonist is attenuated by desensitization, the response parameters will be underestimated if desensitization is not taken into account. Finally, in the presence of desensitization, the relaxation response will fade with time approaching a fixed level of contraction which seems to be independent of the conditions of the system. For example, regardless of the concentration of ISO in this system fractional desensitization was ~80-85%. Clearly a kinetic analysis is essential; the observed rate constant for the processes of relaxation and desensitization would be expected to reflect the characteristics of the system. An exploratory kinetic model was therefore formulated to describe the

relaxation and desensitization components of the ß-adrenergic response separately.

Leff (1986) developed a theoretical model to predict the potential errors in estimation of response parameters by the simultaneous occurrence of receptor desensitization in smooth muscle. In Leff's model, desensitization is described in terms of an irreversible inactivation of a productive agonist-receptor complex (i.e. there is no resensitization in the model). This exploratory model was used to construct simulations of responses under various conditions but was never tested by fitting it to experimental data. In subsequent work (Leff, 1988) the author compared simulated curves with experimental curves which described the time-dependent desensitization of smooth muscle contraction elicited by serotonin (5HT) in the rabbit aorta and guinea pig trachea. However, no attempt was made to estimate the parameters of the model that would fit the data and therefore, only a qualitative comparison between the simulated curves and the experimental curves was shown. The exploratory kinetic model presented here estimates the observed parameters, e.g., levels of response and rate constants, directly from the data. The subsequent exploration of their dependence on the concentration of the agonist provides insight into the possible mechanisms of relaxation and desensitization responses.

The inadequacy of the steady state analysis of the relaxation response to ISO without consideration of ß-adrenergic desensitization is illustrated in the cumulative CRC shown in Fig. 4 and in the inverted U-shaped CRCs that have been previously reported in helically cut thoracic aortic strips from the rabbit (Furchgott, 1953; Fleisch, 1970, Fleisch and Hooker, 1976). It is impossible to distinguish concentration dependent phenomena from time dependent phenomena using such a protocol. Furthermore, no information can be gained concerning the desensitization process. As shown in Fig. 6A, the kinetic characterization that takes into account the desensitization process yields a monotonic CRC for the relaxant effect of ISO (EC_{50} = 0.067 μM). It should be kept in mind that these experiments were carried out in the presence of a fixed concentration of PE; the parameters that describe the CRC for the relaxant effect of ISO have been shown to depend on the concentration of the agonist eliciting contraction (Torphy et al., 1983).

The apparent rate constant for the relaxation response (k_{rel}) shows a saturable dependence on the concentration of ISO (EC_{50} = 0.017 μM). A rate constant reflects the rate limiting step along the pathway for response generation. In a slow-responding system such as the rabbit aorta (i.e. generation of a steady state response is measured in minutes)

it is reasonable to assume that the formation of the drug•receptor complex (D•R) is faster than the generation of the response and therefore, it is not rate-limiting. Thus, the saturable dependence of $\mathbf{k_{rel}}$ on the concentration of ISO suggests that the rate-limiting step is beyond the binding of agonist to the receptor, perhaps the activation of an effector mechanism.

In characterizing the tonic portion of the contractile response to PE in the rabbit aorta, Cory et. al (1984) suggested that PE activated the α-AR to form a D•R which in turn, participates in a slow interaction with an effector which is the rate limiting step. This was indicated by the saturable dependence of the observed rate constant for the onset of the tonic contraction (k_{obs}) on concentration of PE. Thus, the similar dependence of $\mathbf{k_{rel}}$ on the concentration of ISO may also suggest that $\mathbf{k_{rel}}$ represents the interaction of the D•R with an effector to produce the relaxation response. Furthermore, the affinity of the D•R for the effector has been suggested as a molecular definition of drug efficacy (Black and Leff, 1983) and in the case where activation of the effector is rate-limiting, the maximal value of the observed rate constant of a response (k_{obs}^{max}) has been suggested as a kinetic definition of drug efficacy (Cory et al.,1984,1986). Therefore,

it is reasonable to suggest that k_{rel}^{max} may also reflect the efficacy of drugs on the ß-AR system.

One possible explanation for the data presented in this report is that ISO occupies the ß-AR to form D•R and the rate limiting step involves the slow activation of G_s by D•R. Once activated, the α_s subunit dissociates from G_s and activates the catalytic unit of adenylyl cyclase. Adenylyl cyclase catalyzes the conversion of ATP to cAMP which is associated with the relaxation response. This proposed mechanism is consistent with findings from kinetic studies that probed the coupling of the ß-AR to adenylyl cyclase in cells. Tolkovsky and Levitzki used a kinetic approach to examine the mechanism of coupling of the ß-AR to the adenylyl cyclase enzyme in turkey erythrocyte membranes (Tolkovsky and Levitski, 1978, 1981; Tolkovsky, 1983). These studies suggest that the rate limiting step in activation of adenylyl cyclase is the activation of G_s by the D•R complex. In relating the rate-limiting step of the relaxation response in the rabbit aorta to the generation of cAMP in the turkey erythrocyte, we make the assumption that no step beyond the formation of cAMP is rate-limiting.

The kinetic model described in this work also allowed characterization of the ISO induced desensitization. The present study suggests that the observed atypical concentration response curve (Fig 4) is due to the occurrence

of desensitization of the ß-AR. A transient relaxation was seen at every concentration of ISO that produced a relaxation response. This loss of relaxation was not due to degradation of ISO and was mediated by a propranolol sensitive site. The regaining of tension was not due to sensitization of the PE induced contraction and occurred even at concentrations of ISO more than 2 orders of magnitude below reported activity of ISO on the α-AR (Fleisch et. al., 1970). Furthermore, once a dose of ISO (1µM or higher) has produced the biphasic response, no additional dose of ISO would relax the tissue. This attenuation of responsiveness to an agonist is consistent with desensitization.

The magnitude of desensitization (**D**) changes with each concentration of ISO. However, the fraction of the relaxation that is lost due to desensitization (**D/R**) approaches a fixed limit; in this system, ~80-85% of the relaxation response is lost due to desensitization regardless of the concentration of ISO. Thus, in the rabbit aorta preparation, the value of the fractional desensitization cannot be used for the characterization of the desensitization response.

Our results show that there is no dependence of the observed rate constant for desensitization, $\mathbf{k_{des}}$, on the concentration of ISO at concentrations in the range tested (0.03 µM - 10 µM). Thus, in this concentration range, the

desensitization is a zero order process. This might be due to the saturation of ß-ARK at concentrations of ISO below those that could be quantitated in this system. It implies that the K_M for ß-ARK is small relative to the substrate concentration (i.e., the concentration of occupied ß-ARs). Alternatively, zero order kinetics would also be observed if the rate limiting step were the translocation of ß-ARK from the cytosol to the membrane. Such a process was observed as a necessary step in inducing ß-AR desensitization in cells (Strasser et al., 1986b). Because t_d may represent the delay in the onset of desensitization due to a translocation process, it might also explain the lack of dependence of t_d on the concentration of ISO. Another possibility is that desensitization is related to the participation of another kinase, perhaps in addition to ß-ARK, that would not depend on the concentration of ISO but rather on another signal that is generated in the precontracted tissue. Studies to characterize the dependence of k_{des} on the stimulus for contraction are in progress.

In summary, agonist elicited relaxation mediated by the ß-AR and desensitization of the ß-AR can be visualized as time dependent changes in tone in the rabbit aorta. An exploratory kinetic model was developed and used to separate the relaxation and desensitization responses and to characterize the dependence of the ß-adrenergic response

parameters on the concentration of ISO. Kinetic analysis suggests that the rate-limiting step for the relaxation response may be the activation of G_s by the D•R complex which ultimately leads to activation of adenylyl cyclase. It also suggests that the efficacy of a ß-adrenergic agonist may be related to the maximal rate constant for the onset of relaxation. Subsequent reports will further elucidate the possible rate-determining step for the desensitization process which in this system may be related to different intracellular stimuli that activate kinases other than ß-ARK.

Figures for paper I

Figure 1

Computer collected data points demonstrating the protocol for the generation of the biphasic response to ISO in the isolated rabbit aorta. Dots indicate time of drug addition. Contraction was elicited with PE (1 μM) prior to the addition of ISO (1 μM) to elicit the relaxation and desensitization responses. The rate of collection was 1 point every 3 sec for the duration of the trial.

Figure 1

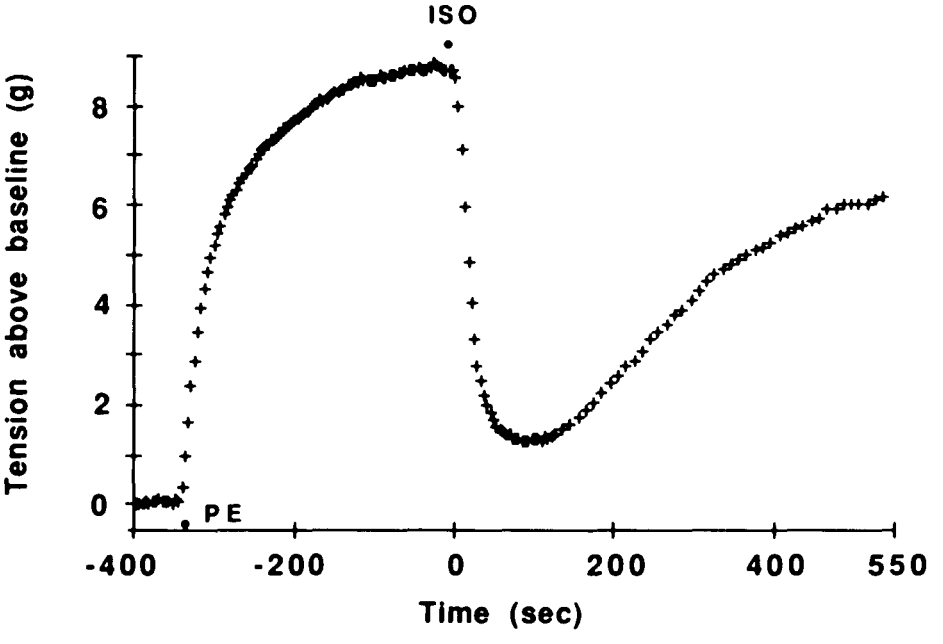

ISO
PE
Tension above baseline (g)
0
2
4
6
8
-400
-200
0
200
400
550
Time (sec)

Figure 2

A. Schematic depiction of the observed and calculated time dependent responses to PE and ISO when the relaxation and desensitization processes appear separated in time (see Methods). The solid curve represents observed changes in tissue tension following the addition of PE and ISO to the organ bath at the dots. When the two processes appear to be temporally separate the calculated magnitudes, **R** and **D** approximate the observed changes in tissue tension. The delay time from the addition of ISO to the onset of desensitization is defined as $\mathbf{t_d}$. The level of contraction from which the desensitization process begins (i.e. the plateau), **P** is obtained directly from the tracing.

B. Schematic depiction of observed and calculated time dependent responses to PE and ISO. The solid curve represents observed changes in tissue tension following the addition of PE and ISO to the organ bath at the dots. The dotted curves represent the calculated responses that result from mathematical separation of the relaxation response from the desensitization response (see text). **C** (g) is an observed parameter that represents initial contraction while **R**(g) and **D**(g) are the calculated magnitudes of the relaxation and desensitization processes respectively. The rate constants of the two processes are $\mathbf{k_{rel}}$ (min^{-1}) and $\mathbf{k_{des}}$ (min^{-1}).

Figure 2A

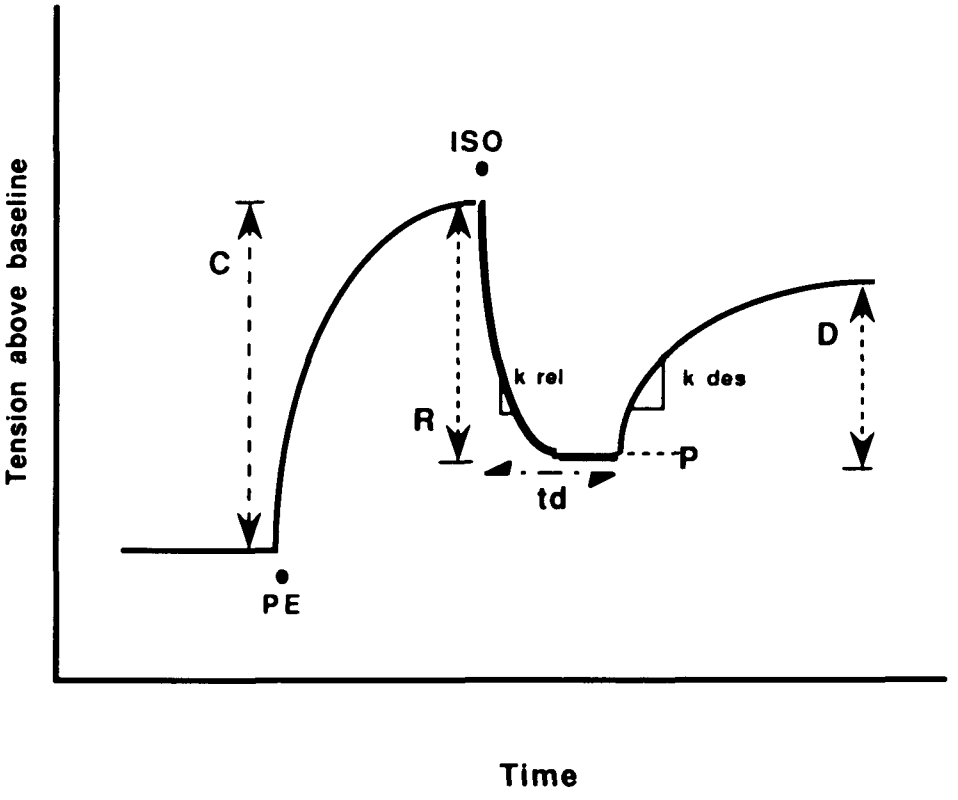


Figure 2B

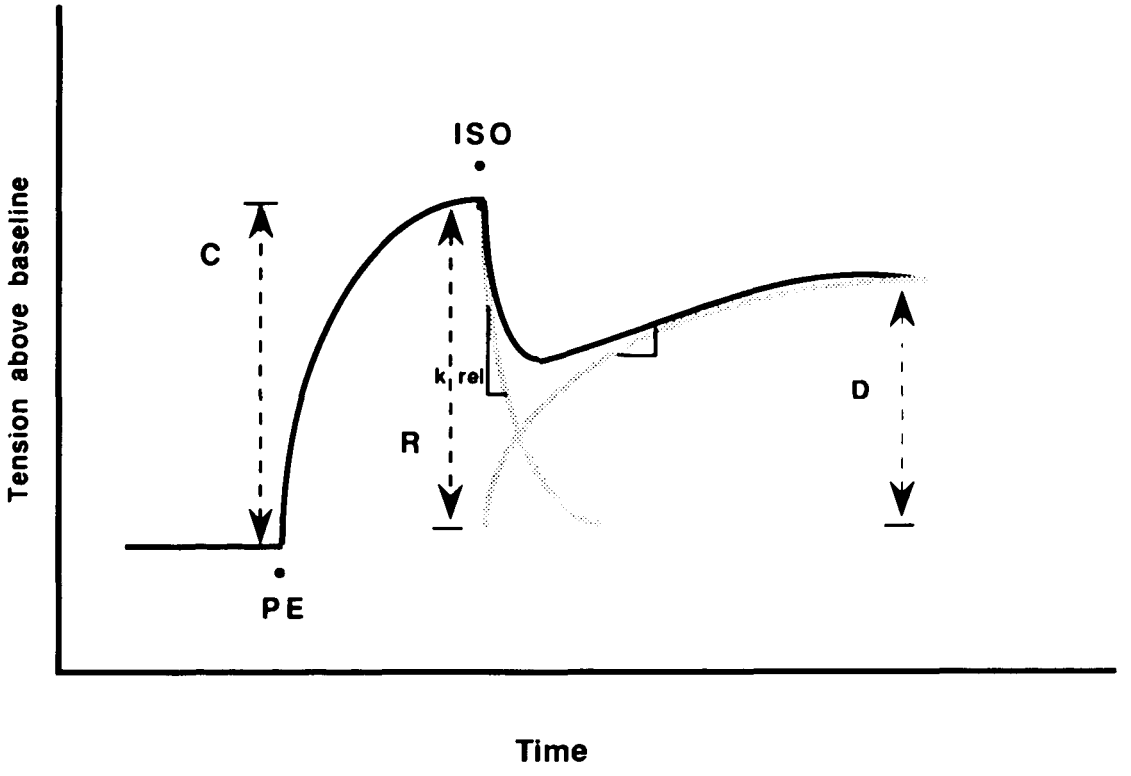


Figure 3

A representative biphasic response to ISO (0.2 μM) in the presence of PE (1 μM; precontraction not shown). The first 61 points of the curve were fitted to Eq. (2) (see Methods): R= 4.8 g, k_{rel} =0.037 sec^{-1} r^2=0.99. The final 41 points of the curve were fitted to Eq (3): D= 4.8 g, k_{des} = 0.0036 sec^{-1}, t_d=237.011 sec. r^2 = 0.99. The middle points (in the shaded area) were not used.

Figure 3

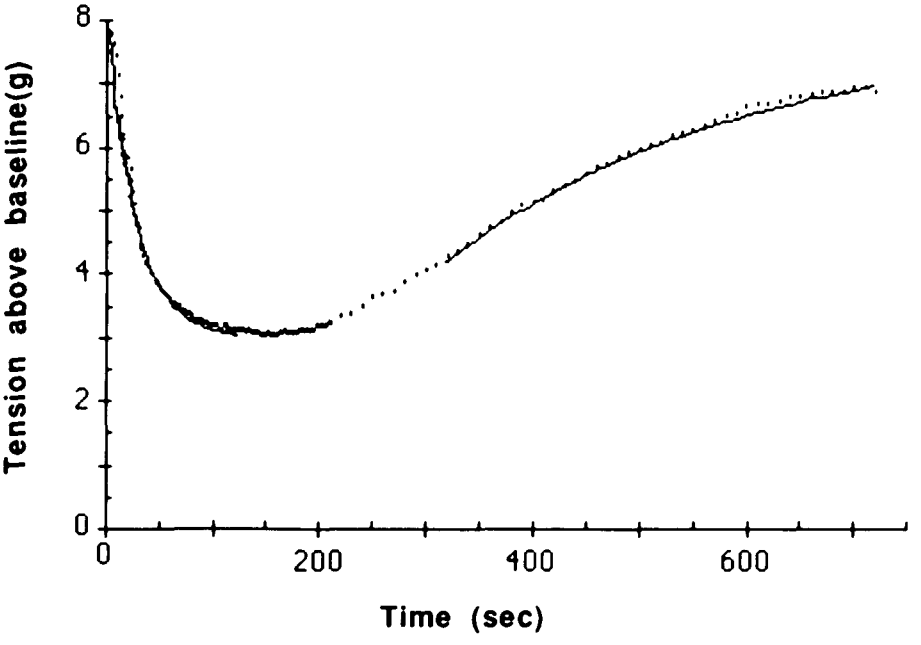


Figure 4

Representative tracing of an original polygraph recording demonstrating the production of a cumulative CRC to ISO in the presence of PE (1 μM). ISO was added to the bath to create cumulative concentrations (in μM) indicated at each dot. Similar results were obtained in 9 repetitions from 5 animals.

Figure 4

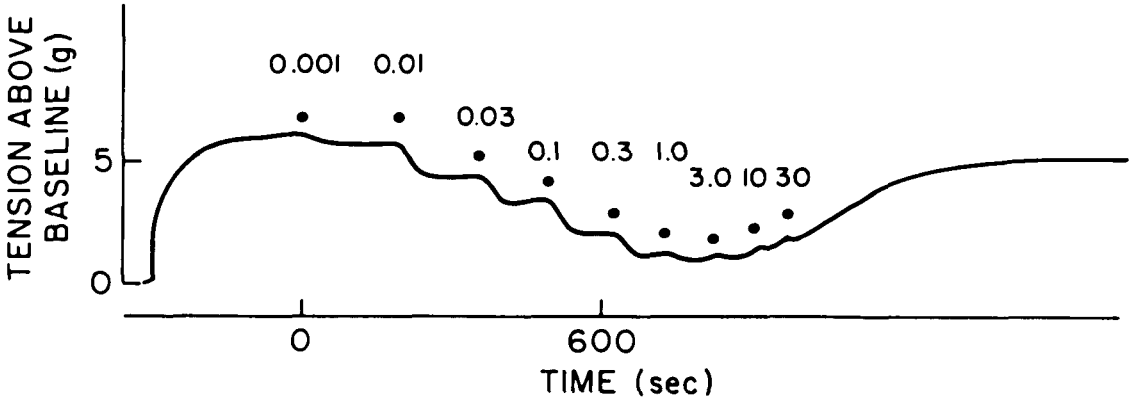

TENSION ABOVE BASELINE (g)
5
0
0.001
0.01
0.03
0.1
0.3
1.0
3.0
10
30
0
600
TIME (sec)

Figure 5

A characteristic kinetic profile of the responses to different concentrations of ISO in a ring of rabbit aorta in which tension was produced by PE (1 μM; precontraction not shown). The drugs were washed out after the tissue tension reached steady state and the tissue was allowed to relax for 60 minutes to allow for resensitization before the next addition of PE. ISO concentrations for the production of each curve are listed above each curve. The order of testing ISO concentrations was randomized. Points shown were digitally recorded using an Apple IIe computer (see Methods). Similar experiments were performed on 22 rings of aorta from 8 animals.

Figure 5

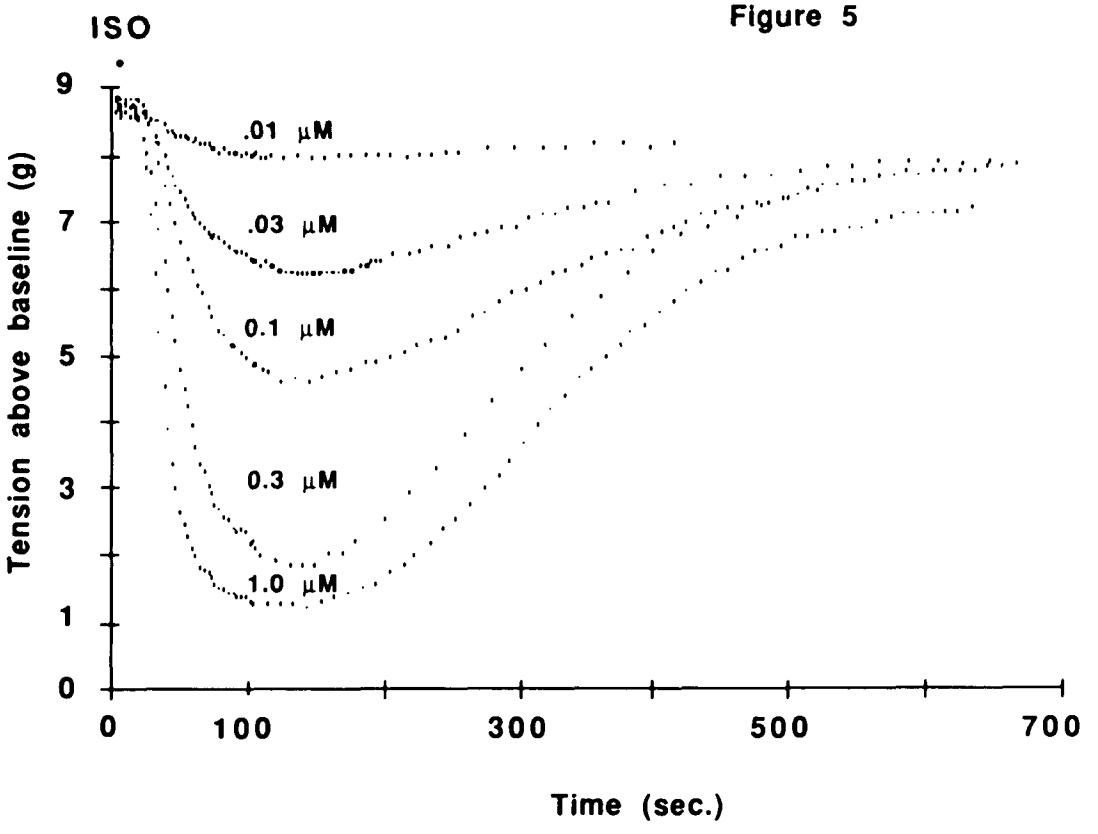
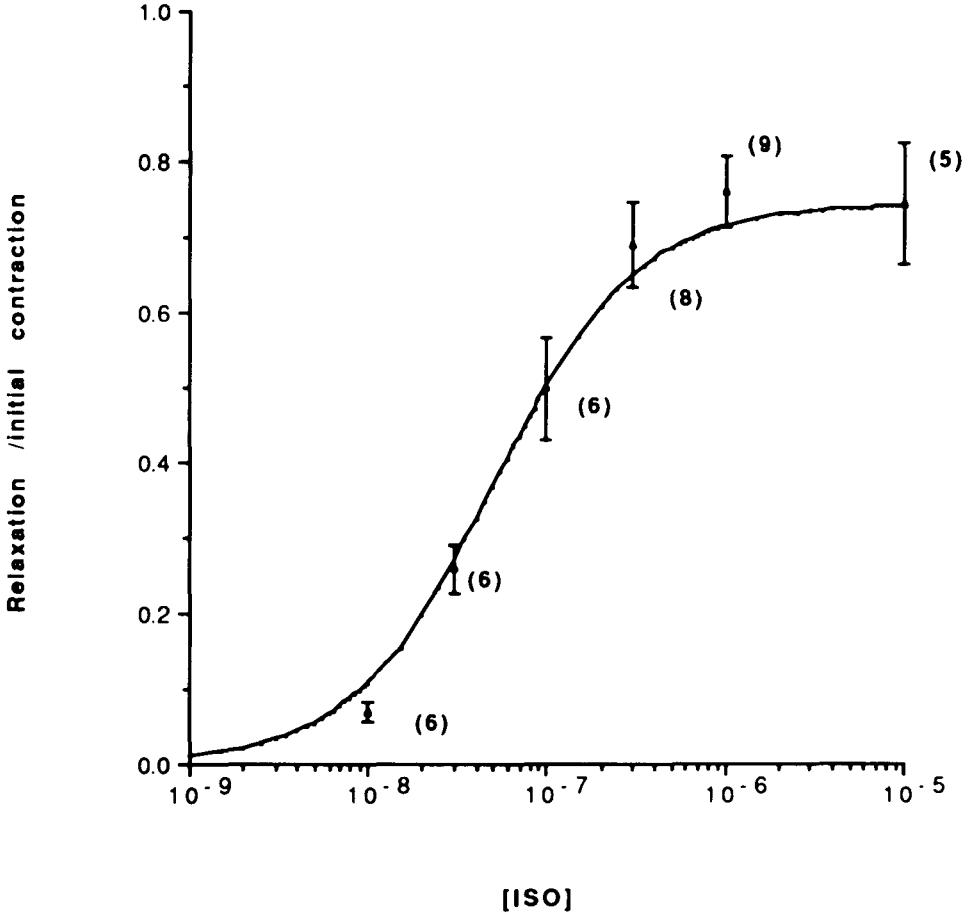

ISO
9
7
5
3
1
0
Tension above baseline (g)
.01 µM
.03 µM
0.1 µM
0.3 µM
1.0 µM
0
100
300
500
700
Time (sec.)

Figure 6

6A: A representative log concentration response curve for the fractional relaxation (**R/C**) produced by ISO in the rabbit aorta. The rings were precontracted with PE (1 μM). Each point represents the mean ±SEM (n=16 repetitions from 6 rabbits). The curve, obtained from fitting the mean data points to equation 1 has an EC_{50} of 0.067μM, an Emax of 0..75 and a slope index of 1.14.

Figure 6A


Relaxation /initial contraction
1.0
0.8
0.6
0.4
0.2
0.0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
(6)
(6)
(6)
(8)
(9)
(5)
[ISO]

6B: The dependence of the rate constant for the onset of the relaxation response (k_{rel}) on [ISO]. Each point represents mean ± SEM (n= 16 repetitions from 6 rabbits). The curve obtained from fitting the data to Eq (4) has an EC_{50} of 0.017μM, an k_{rel}^{max} of 0.044 sec^{-1} and a slope index of 0.98. The point at 10μM ISO was omitted from the fitting procedure.

Figure 6B

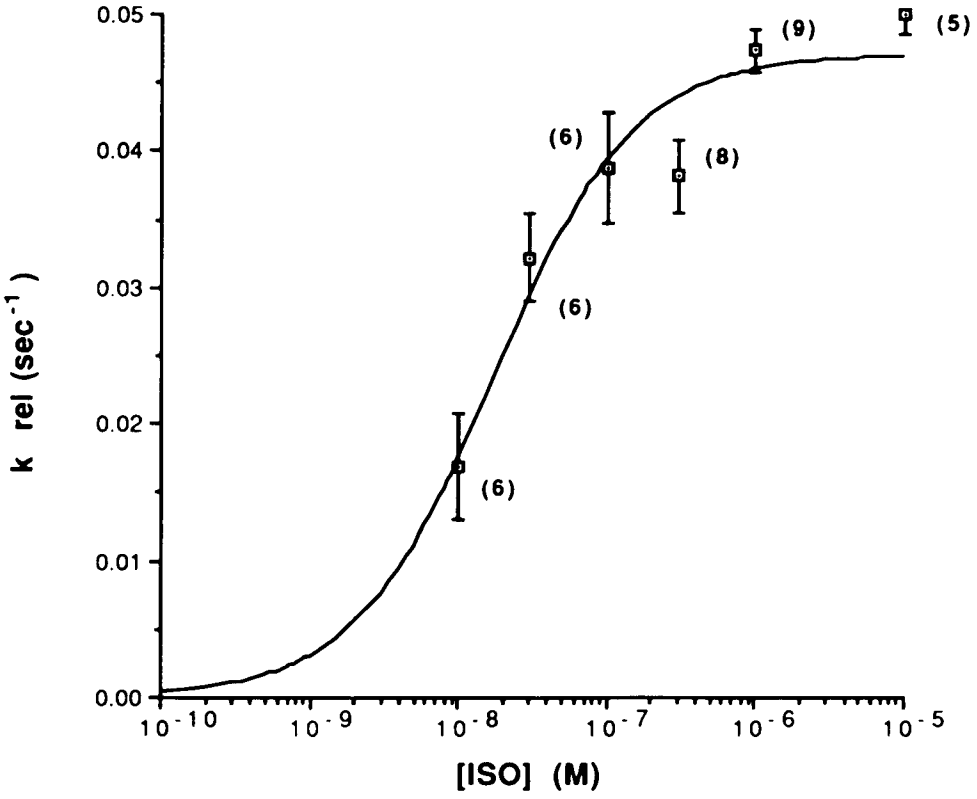
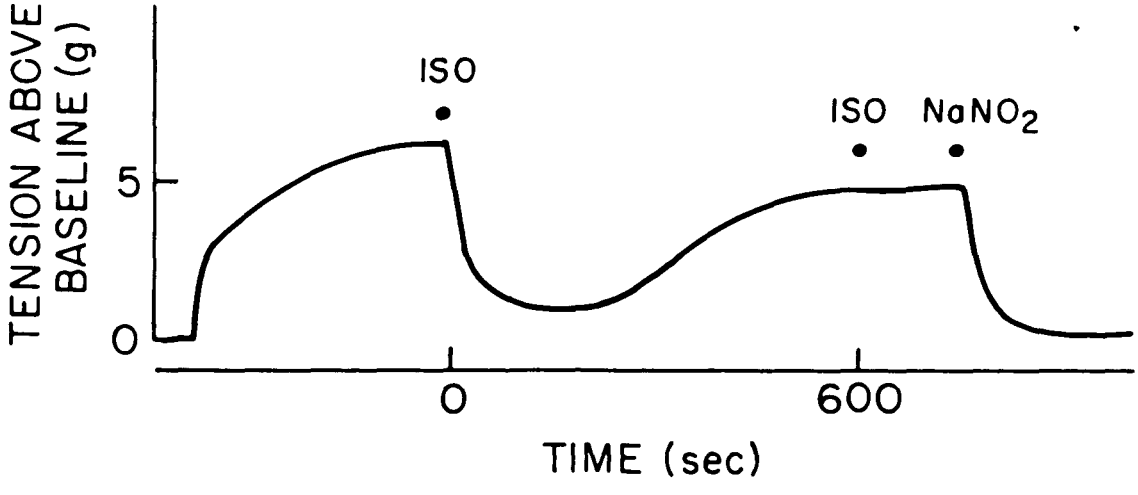

0.05
0.04
0.03
0.02
0.01
0.00
k rel (sec^{-1})
(9)
(5)
(6)
(8)
(6)
(6)
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
[ISO] (M)

Fig. 7

Representative tracing of an original polygraph recording of an aortic ring precontracted with PE (1 μM) and relaxed with ISO (10 μM) demonstrating the lack of response to a second addition of ISO (10 μM; n=8 repetitions from 3 rabbits) Similar results were obtained when the first concentration of ISO was 1μM (data not shown; n=5 repetitions from 2 rabbits). The subsequent addition of 4 mM $NaNO_2$ to the preparation led to stable relaxation of the tissue (n=2 repetitions from 2 rabbits).

Figure 7



paper II

Functional Interactions in Smooth Muscle: The effect of contractile agonists on the kinetics of ISO-induced relaxation and desensitization.

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Functional antagonism in the rabbit aorta preparation was initially described as ISO induced relaxation of precontracted tissue (Furchgott and Bhadrakon, 1953; Fleisch et al., 1970). A kinetic analysis of the response to isoproterenol (ISO) in aortic strips precontracted with phenylephrine (PE) has recently been presented (Keitz et al., paper I). Under these conditions, the response to ISO was biphasic and consisted of a relaxation followed by a regaining of tension which was identified as desensitization. Due to the simultaneous occurrence of these two processes, a kinetic analysis of the time-dependent responses was necessary to separate the relaxation process from desensitization.

The kinetic approach enabled a full characterization of the two observed responses, i.e., relaxation and desensitization and the dependence of the parameters on the concentration of ISO. The relaxation response parameters showed a saturable dependence on the concentration of ISO. The parameters that describe the desensitization response showed no apparent dependence on the concentration of ISO. However, the previous work was done at a constant concentration of PE and there are numerous reports that some of the parameters that describe the relaxation response to ISO in tracheal smooth muscle (i.e. EC_{50} and E_{max}) depend on the concentration and identity of the contractile agents (Van Den

Brink, 1973; Russell, 1984; Torphy et al., 1986; Madison et al., 1989; Buckner and Saini, 1975; Torphy, 1983). Thus we explored the dependence of the relaxation response to ISO on the concentration and identity of the contractile agent. Contraction was elicited by activating either the α-AR or $5HT_2$ receptor with PE or 5HT respectively.

We have previously reported that neither the fractional desensitization nor the observed rate constant for desensitization were dependent on the concentration of ISO in the range of 0.03μM to 10 μM ISO (Keitz et al., paper I). One possible explanation for this lack of dependence under these conditions is that the desensitization process is modulated primarily by a stimulus generated by the contractile agent rather than by the stimulus generated by occupancy of the ß-AR. Therefore, in the current work we explored the dependence of the desensitization response parameters on the concentration of the contractile agent.

METHODS.

Tissue preparation.

Male New Zealand White Rabbits weighing between 1.4-1.8 kg. (Ace Animals, Inc., Boyertown PA) were sacrificed by CO_2 asphyxiation and the thoracic aortae were rapidly excised and prepared as previously described (Keitz et al., paper I). The tissues were cut into 4-6 rings 0.5-0.6 cm in width which were suspended between stainless steel hooks in 20-ml organ baths. The baths contained Krebs-bicarbonate buffer bubbled with 95% O_2-5% CO_2 to maintain a pH of 7.4 ± 0.2 at 36° ± 1 °C. The tissues were initially set to ~2 g tension and allowed to relax until a stable tension was maintained. The minimum stabilization time was 1 hour. Once resting tension was stabilized, baseline tension was set to a force between 1 and 2 g. All rings were primed twice by eliciting a contraction with 10 μM PE. Drugs were removed from the bath by draining and replacing the buffer.

Solutions.

Krebs-bicarbonate buffer contained the following components (in mM) in glass distilled water: NaCl, 110; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 2.35; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11; Na_2EDTA, 0.03. For those trials in which contraction was elicited with 40 mM KCl, the following components of the Krebs-bicarbonate buffer were modified (in mM): NaCl, 74.8; KCl, 40.

Chemicals.

5-HT·HCl (5HT); isoproterenol phosphate (ISO) [Sigma, St. Louis, MO]; All other chemicals were of analytical grade as previously reported (Clancy, 1985).

Responses to ISO.

In order to test the effects of varying the nature of the agonist eliciting contraction on the ß-adrenergic response parameters, responses to ISO were obtained in the presence of equiactive doses (i.e., doses that elicit the same increase in tension) of PE, 5-HT, HA or KCl (Fig. 1). The responses to PE, 5HT and KCl were stable for the duration of the trial, whereas the contraction induced by HA showed loss of tension with time. Furthermore, the dependence of the contraction on the concentration of KCl was very steep resulting only in threshold or maximal

contractions by manipulation of the KCl in the buffer. Thus, a full characterization of ISO responsiveness in the presence of varying concentrations of KCl could not be observed. Therefore, the dependence of the parameters of the response to ISO on concentration of the agonist eliciting contraction was studied only for PE and 5HT. The order of testing various concentrations of PE or 5HT was randomized. At the end of the desensitization, when tissue tension reached steady state, the drugs were washed out by replacing the buffer three times and the tissue was left to rest for one hour to allow for complete recovery of responsiveness.

The ß-adrenergic response parameters for 0.3 μM ISO were determined at concentrations of either PE or 5HT ranging from 0.03μM to 10μM. The effect of varying the concentration of PE on the ß-adrenergic response parameters on the concentration of PE or 5HT was compared to that of varying the concentration of 5HT over a similar range of occupancies expressed as the ratio of [drug]/K_A for each agonist. The range of values of [drug]/K_A for PE was ~0.1 to 40 and for 5HT the range was ~0.4 to 100. The values for the K_A's were determined in the rabbit aorta preparation (Christ et al. , in press). The mean effect, E, was determined for each concentration of drug, D, and the

mean values were fit to the logistic equation for estimation of EC_{50}, E_{max} and slope indices (n):

$$E = E_{max}/(1+(EC_{50}/[D])^{n}) \qquad (1)$$

Collection of data points.

Isometric contractions were measured with Grass FT03C force displacement transducers connected to the Grass polygraph model 7D. Data points corresponding to tension were collected at a frequency of 0.04 - 1 Hz and stored on floppy disk for subsequent analysis as was previously described (Keitz et al., paper I). Digital values of muscle tension were transferred to a Sun 3/110 workstation for non-linear analyses with the PROPHET II computer program.

The kinetic model:

The relaxation and desensitization responses to ISO were separated using a model previously described (Keitz et al., paper I). The relaxation response was modeled as an exponential decay:

$$T = C - R\,(1 - e^{(-k_{rel} * t)}) \qquad (2)$$

where **T** is the observed tissue tension, **C**-the initial contraction, **R** the magnitude of the ISO induced relaxation, k_{rel}-the observed rate constant for relaxation and t- the time elapsed since the addition of ISO. **C** was measured from the experimental curve immediately before the addition of ISO to the bath. The values for **R,** and k_{rel} were estimated by

fitting experimental data to this model with a non-linear regression procedure.

The desensitization response was also modeled as a simple exponential increase in tension:

$$T = P + D(1- e^{(-k_{des} * (t-t_d))}) \qquad (3)$$

where **T** is the observed tissue tension, **P** is the plateau from which the regaining of tension begins, **D** is the magnitude of the ISO induced desensitization, k_{des} is the observed rate constant for desensitization and t_d is time following the addition of ISO before desensitization is observed. The valid domain of equation 3 is only for $t \geq t_d$. The values of **D**, k_{des}, and t_d were estimated using a nonlinear fitting procedure whereas the plateau, **P**, was measured.

When t_d approaches zero (i.e. when the desensitization response begins before the relaxation response has reached its maximum) the entire tracing must be described by a single equation in which **P** is replaced by eq.2.

$$T= C - R (1-e^{(-k_{rel} * t)}) + D (1-e^{(-k_{des} * t)}) \qquad (4)$$

All of the data were evaluated initially with Eq. 2 and 3. When td was $\leq$ 5 times the half-life for relaxation ($T_{1/2} = \ln 2/k_{rel}$), all the parameters were recalculated using equation 4 and these values were used for subsequent analysis. In the fitting, t_d was constrained to be ≥ 0 and R was constrained to

be ≤ C. In order to compare different rings, the extent of relaxation, is described as fractional relaxation, **R/C**. Likewise, the desensitization is represented as fractional desensitization, **D/R**.

RESULTS.

ISO (1 μM) induced both a relaxation and desensitization response in all rings precontracted by four different agents: PE (n = 54 repetitions from 16 animals), 5HT (n=42 repetitions from 14 animals), HA (n=12 repetitions from 2 animals) or KCl (n = 24 repetitions from 5 animals). However, as shown in Fig. 1, the prototypical responses for each of these contractile agents appeared quantitatively different. The contraction elicited by HA faded within the time it took to complete a trial and the response curve to KCl was too steep to allow full characterization of the ISO responses over a range of contractions. Therefore, the dependence of the ISO response parameters on the concentration of the contractile agent was only studied in tissues precontracted with PE or 5HT.

Table I summarizes the characteristics of ISO induced relaxation and desensitization in the rabbit aorta precontracted with 1 μM 5HT. For the purpose of comparison, the same characteristics obtained in the presence of 1 μM PE as the contractile agent are presented in the table as well. Clearly, the behavior of the ß-adrenergic response parameters as a function of the concentration of ISO is similar regardless of whether the rings were precontracted with 1 μM PE or with 1 μM 5HT. Specifically, in both cases the parameters that describe the relaxation response, i.e., fractional relaxation and

k_{rel}, were saturable with respect to ISO concentration, while the parameters that describe the desensitization response, fractional desensitization, i.e., k_{des} and t_d, were insensitive to changes in the concentration of ISO. It bears emphasis that the apparent commonality between ISO response parameters in the presence of PE or 5HT lies in the similar trends rather than in the numerical values of the parameters as these values will change by altering the concentration of the contractile agonist (Torphy, 1983).

ISO induced relaxation: dependence on the concentration and identity of the agonist eliciting contraction.

A representative series of relaxation responses to ISO in a ring precontracted with different PE concentrations is shown in Fig. 2. The desensitization response for this series of curves is not shown because the t_d was > 5 $(t_{1/2})$rel, and thus the relaxation response was not contaminated by the simultaneous desensitization which could be described by equation 2 (see Methods). This representative example, is characteristic of the general behavior; both the fractional relaxation (**R/C**) and the observed rate constant for relaxation (k_{rel}) decrease with increasing occupancy of the α_1-AR (see inset in Fig. 2).

Fig. 3 illustrates the relationship between the mean fractional relaxation induced by ISO and the concentration of the contractile agent, PE or 5HT, expressed as multiples of its K_A's. There is a clear difference between the fractional relaxation produced by ISO in the presence of 5HT and that observed in the presence of PE. ISO produces ~80% relaxation at a 5HT concentration that correspondes to 28% occupancy of the $5HT_2$ receptor. However, even at 99% occupancy of the $5HT_2$ receptor, the relaxation remained greater than 70%. . In contrast, ISO induces an almost complete relaxation at 25% occupancy of the α-AR. However, as the occupancy increases to 98%, the fractional relaxation is diminished to 31%.

The rate constant for relaxation, $\mathbf{k_{rel}}$, shows a less dramatic dependence on the occupancy of the α_1-AR and $5HT_2$ receptor. As shown in Table II, increasing the concentration (occupancy) of PE or 5HT reduced the rate constant. These conclusions are supported by one way Analysis of Variance (ANOVAR) with repeated measures of $\mathbf{k_{rel}}$ from consecutive trials on the same aortic ring which was significant ($p \leq 0.005$). All subsets of data from a total of 24 rings from 6 animals that were tested by ANOVAR showed a significant change in k_{rel} with concentration of the contractile agent ($p \leq .0.05$).

ISO induced desensitization: dependence on the concentration and identity of the agonist eliciting contraction.

The fractional desensitization (**D/R**) does not change as a function of the occupancy of the α_1-AR or $5HT_2$ receptor. The average **D/R** is 0.93 ± 0.055 in the presence of PE (n = 50 repetitions from 6 animals) and 0.81 ± 0.03 in the presence of 5HT (n = 62 repetitions from 6 animals) even though the [drug]/KA was varied from 0.3 to 40 for PE and 0.3 to 100 for 5HT. However, at the lowest concentration of 5HT when the starting tension was small prior to the addition of ISO ([drug]/KA <0.3; data not shown), the regeneration of tension following the ISO induced relaxation often exceeded the starting tension (i.e., **D/R**>1).

In contrast, the rate constant for desensitization, $\mathbf{k_{des}}$, was dependent on the [drug]/K_A in a saturable manner when the tissue was contracted with either PE or 5HT. The dependence i shown in Fig. 4, however, the values of [drug]/K_A at which kdes reaches half its maximal value cannot be considered different for the two curves because the 95% confidence intervals for the values of midpoints (expressed as EC_{50}/K_A for PE or 5HT) overlap.

The dependence of $\mathbf{t_d}$ on occupancy of the contractile receptor is shown in Fig. 5. The $\mathbf{t_d}$ shows a dependence on

occupancy, i.e., on [drug]/K_A. The t_d decreases by approximately fourfold as the concentration of PE is increased from 0.4 to 13.5 times its K_A for the α_1-AR. Much less change in t_d occurs over a similar range of 5HT concentrations where the decrease is only approximately twofold.

DISCUSSION.

Concentration Response Curves to ISO in rings precontracted with PE or 5HT: The functional antagonism produced by ISO on various tissues was precontracted with PE or 5HT is qualitatively similar. In the presence of either PE or 5HT, the relaxation parameters displayed saturable dependence whereas the desensitization parameters were independent of ISO concentration. This similarity suggests that PE and 5HT produce similar contractile stimuli that are functionally antagonized by ISO. Such a conclusion is consistent with the many commonalities that have been reported between the contractions elicited by activation of either the α_1-AR or the $5HT_2$ receptor. For example, both PE and 5HT induce a transient phasic contraction that is followed by a sustained tonic contraction in the rabbit aorta. In both cases, the phasic contraction is associated with the release of intracellular calcium stores while the tonic contraction is due to increase in the influx of calcium (Khalil and vanBreeman, 1988; Cory et al., 1984, 1986). Furthermore, both the underlying mechanism of these contractions for both α-adrenergic agonists (Abdel-Latif, 1986) and 5HT agonists (Roth et al., 1986) is related to the agonist-stimulated turnover of phosphotidylinositol in smooth muscle preparations. There is accumulating evidence that PI turnover is the consequence of the activation of

phospholipase C. The mediating species between the receptor and PLC is Gs. Thus, the saturable dependence of the rate constant for relaxation on the concentration of ISO may suggest that the rate-limiting step for the relaxation process is activation of an effector, perhaps activation of G_s by the D•R complex (Keitz et. al, paper I).

The lack of dependence of the desensitization parameters on the concentration of ISO is inconsistent with the hypothesis that ß-ARK mediates desensitization in this system (Keitz et al., paper I). This lack of dependence was seen in rings precontracted with either PE or 5HT and is consistent with the hypothesis that a kinase other than ß-ARK is mediating desensitization in this system.

Fractional relaxation induced by ISO: dependence on the agonist for contraction: Functional antagonism, demonstrated as the ISO induced relaxation of precontracted aortic rings, shows an inverse relationship to the concentration of the contractile agent. Thus, in the presence of increasing concentrations of PE, the relaxant effect of ISO decreases. Similar behavior was reported in isolated airway preparations (Van den Brink, 1973; Buckner and Saini, 1974; Torphy, 1983). For example, Torphy et al. (1983) showed that the EC_{50} of the relaxant response to ISO increased by 500 fold when the concentration of methacholine was increased from 0.03 µM to

3 μM. The relaxation to ISO could be abolished completely by increasing the concentration of methacholine to 30 μM.

This inverse relationship can be understood in terms of Mackay's model for functional interactions (1981). Mackay describes the events that translate receptor occupancy into response as a chain of stimuli with the "final" stimulus being the events in the system that are directly responsible for the observed state of the cell or tissue. When more than one receptor is activated, each produces a chain of stimuli that may interact at a number of points along the stimulus chain, the simplest interaction being an additive one, although non-additive interactions are also possible.

In the rabbit aorta preparation, the state that is measured is tissue tone in grams of tension above baseline. Each level of tone is associated with its own "final" stimulus which is the result of an interaction between the chain of stimuli for contraction initiated by PE and the chain of stimuli for relaxation initiated by ISO. One possible interaction between the stimuli elicited by PE and ISO is presented schematically in figure 6.

In an additive model, the arrows in Fig. 6 would represent an additive interaction and the summation of stimuli would determine the level of tissue tone. The shape of the relaxation will depend on the stimulus for contraction and its

interaction with the stimulus for relaxation. Indeed, the dependence of the fractional relaxation on the concentration of 5HT was different from the dependence observed with PE over a similar range of occupancies (see Fig. 3). This is consistent with previous reports that the relaxant effect of ISO depends on the identity as well as concentration of the agonist eliciting the contractile response (Torphy et al., 1983; Van Amsterdam et al., 1989). Specifically, in tracheal smooth muscle, ISO or PGE_2 induced smaller fractional relaxation at maximal concentrations of the muscarinic agonists, acetylcholine and methacholine and higher fractional relaxation at maximal concentrations of HA, 5HT and leukotriene D_4 (Russell, 1984; Torphy, 1984; Madison et al., 1989; Van Amsterdam et al., 1989).

In view of Mackay's model, if the functional interaction can be considered a summation of stimuli, then the antagonistic interaction between stimuli affects the relaxation response but also affects the contractile response. Therefore, the difference in the ISO induced fractional relaxation could be explained by a differential effect of ISO on the contraction produced by PE vs 5HT. For example, ISO might induce a greater decrease in the efficacy of 5HT than in the efficacy of PE. Thus, in the presence of ISO, 5HT is converted to a partial agonist, whereas PE remains a full agonist.

Therefore, the fractional relaxation would be greater in the presence of 5HT than PE.

Figure 3 can be considered a CRC to PE or 5HT in the presence of a single concentration of ISO. At very high concentrations of PE, the fractional relaxation is small or the contraction is altered little by the addition of ISO. The relaxant effect of ISO has been shown to approach zero at very high concentrations of PE (Torphy et al., 1983). The CRC to PE reaches its maximum when the contraction reaches its original level without any ISO. Thus, the CRC to PE in Fig. 3 represents a shift to the right with little change in the maximal response (the reported EC_{50} for PE induced contraction in the rabbit aorta in the absence of ISO is 0.24 μM or 0.33 x K_A; Christ et al., in press). Alternatively, the CRC to 5HT shown in the same figure shows a decrease in the maximal response, which seems to be saturated at ~30% remaining contraction (the reported EC_{50} for 5HT induced contraction in the rabbit aorta in the absence of ISO is 0.074μM or 0.28 x K_A; Christ et al., in press). This possibility could be tested by the production of full CRCs to 5HT and PE in the presence of increasing concentrations of ISO. We would predict on the basis of this hypothesis that the CRC to PE would simply shift in parallel to the right, whereas the CRC to 5HT would be shifted downward.

Alternatively, the differences in ISO induced fractional relaxation could be explained by several different mechanisms: 1- A second messenger involved in the contractile response that is generated by the activation of the α_1-AR or $5HT_2$ receptor might play a role in the relaxation of precontracted tissue. The differential production of this second messenger by α-adrenergic or 5HT agonists might correlate with the differential relaxant effect of ISO in rings precontracted with PE or 5HT. For example, Van Amsterdam et al. (1989) showed that varying the concentration of histamine or muscarinic agonists produced alterations in the ISO-induced relaxant response. These alterations in the response to ISO were better correlated with changes in PI metabolism than with level of contraction. 2- A mediator that is generated by the activation of the α_1-AR or $5HT_2$ receptor but that does not play a role in the contractile process might alter the ISO relaxant effect. For example, activation of both the α_1-AR and $5HT_2$ receptor have been associated with the stimulation of prostacyclin release in smooth muscle preparations including strips of saphenous vein as well as rabbit aorta smooth muscle cells in culture (Boeynaems et al., 1987; Kokkas and Boeynaems, 1988). Furthermore, Hiraffuji et al. (1987) showed that 5HT produced prostacyclin release at a lower range of occupancies than norepinephrine in the rat aorta smooth muscle preparation. If

prostacycline was involved in producing a change in the response to ISO, then the differential production of this messenger by PE vs 5HT might account for the observed differences. 3- PE or 5HT may activate receptors which interact directly with the adenylyl cyclase enzyme and thereby either increase or decrease the relaxant effect of ISO. Although there is evidence in the literature that suggests that the differences in the relaxation response to ISO could be explained by a direct interaction (either positive or negative coupling) of the receptor mediating contraction with the adenylyl cyclase system (Torphy et al., 1985, Jones et. al, 1987; Trevethick et al., 1984, 1986; Sumner et al., 1989), it is unlikely that this interaction is responsible for the differences in ISO responsiveness in the rabbit aorta. The receptor population in the rabbit aorta was suggested to be homogeneous $5HT_2$ (Clancy et al., 1985) which is not known to be coupled to AC in this system. Although there is a small population of α_2-AR in the aorta which is negatively coupled to AC, PE is thought to be a relatively selective agonist for the α_1-AR. Furthermore, activation of the α_2-AR in the aorta has been shown to contract the tissue by a mechanism that is not well understood.

The rate constant for relaxation: dependence on the agonist for contraction: In the present study, changes in $\mathbf{k_{rel}}$,

a kinetic parameter, were described over a range of PE or 5HT concentrations in addition to changes in the fractional relaxation (table II). Although the dependence of fractional relaxation on the concentration of agonist for contraction was different for PE than for 5HT, the dependence of k_{rel} on the concentration of contractile agonist was the same for 5HT and PE. When the concentration of ISO eliciting relaxation was kept constant (0.3 μM ISO) and the occupancy of the α_1-AR or $5HT_2$ receptor was increased, the k_{rel} decreased. If it is assumed that the number of ß-ARs does not change and the affinity of ISO for the ß-AR is not altered by PE or 5HT, then any change in the ISO response must reflect a change in the efficacy of the response.

The rate constant for the onset of contraction, k_{obs}^{max} was shown to be related to efficacy (Cory et al. 1984, 1986). Similarly, k_{rel}^{max} may have the same relationship. Although, this work was not performed at k_{rel}^{max}, the relationship between efficacy and k_{rel} is through a saturable concentration dependence. Therefore, the changes in k_{rel}^{max} should reflect changes in efficacy. If k_{rel}^{max} is related to an efficacy term, then a decrease in the efficacy of the response would result in a downward rather than parallel shift in the ISO CRC.

We wanted to examine if an additive model could explain the finding that there was an inverse relationship between the rate constant for relaxation and the concentration of agonist eliciting contraction. A complete, time dependent description of the functional interactions that occur in this complex system would require four-dimensions because the tension that we observed depends on the following variables: 1-the stimulus for contraction that is elicited by PE or 5HT prior to the addition of ISO; 2- the stimulus for relaxation; and 3- time. Because in our experiments the stimulus for relaxation remained constant, a three-dimensional plot should suffice (illustrated schematically in Fig. 7).

A hyperbolic relationship between tone and the. stimulus for contraction is depicted on one set of the axes (Black and Leff, 1983). The saturable muscle tone depends on the increase in the stimulus for contraction which reflects the increase in the concentration of the contractile agonist (PE or 5-HT). While the figure shows a specific relationship between tone and the contractile stimulus, the hyperbolic relationship is of a general nature and should hold for a combined stimulus such as the one arising from the formulation of functional antagonism (Mackay; see Fig. 6). In such a case the resulting stimulus is the algebraic sum of the individual opposing stimuli for contraction and for relaxation. Specifically, in the experiments described in this work,

at a particular stimulus for contraction the tissue reaches a steady state tone before the addition of ISO. The subsequent addition of ISO leads to a time dependent relaxation illustrated in the other set of axes as a decrease in tone with time. This would correspond to a time dependent development of the relaxing stimulus which functionally antagonizes the constant contractile stimulus. The relaxation is shown for three different levels of tone which correspond to three different stimuli for contraction. Consistent with our results (see above) the relaxation is slowest at high stimuli and becomes faster as the stimulus decreases.

Because of the hyperbolic relationship between tone and stimulus the rate of relaxation (i.e., the change in tone with time) will show an inverse dependence on the total stimulus and a direct dependence on the rate of its change. As the concentration of the contractile agonist increases the stimulus for contraction increases and its contribution to the total stimulus becomes larger. Since at each concentration of the contractile agonist the stimulus for relaxation is constant the apparent rate constant for relaxation should show an inverse dependence on the stimulus for contraction Indeed, this dependence is reflected in the apparent rate constant for relaxation that was measured in this work.

Desensitization of the ß-AR: dependence on the stimulus for contraction: The current work confirms our previous

observations that the fractional desensitization, the steady state measure of the desensitization approaches a fixed limit which is independent of the concentration of either the contractile or the relaxing agonist (Keitz et al., papers I and II). Therefore, the steady state measure of desensitization is not an appropriate parameter to characterize the desensitization response.

In contrast to the steady state measure of desensitization, the two kinetic parameters, t_d and k_{des}, were both dependent on the concentration of the contractile agonist in a saturable manner. Because the t_d is the time for the onset of desensitization, its inverse will reflect an apparent rate constant that describes this process. It follows that both the rate constants for the onset and for the development of ß-adrenergic desensitization response depend on the activation of the α_1-AR or the $5HT_2$ receptor. In the presence of either PE or 5HT, the t_d decreased (i.e. the apparent rate constant increases) to a limiting value with increasing concentration of the contractile agonist. Furthermore, the curve that describes the dependence of t_d on the concentration of 5HT is to the left of the curve that describes t_d as a function of PE concentration. The relative positions of the curves that describe k_{des} as a function of the concentration of PE or 5HT are the same as for t_d. These curves suggest that 5HT is more

efficacious than PE in both initiating and facilitating desensitization of the ß-AR.

The occurrence of a lag-time preceding the onset of a response and the saturable dependence of that lag time on the concentration of an agonist was previously described by Tolkovsky and Levitsky (1978, 1981, 1982) who studied the AC-linked ß-AR in turkey erythrocytes. Tolkovsky and Levitsky showed that the lag time for the accumulation of cAMP and the observed rate constant for the onset of the cAMP accumulation were dependent on the concentration of ISO in a saturable manner. Furthermore, these authors also demonstrated a linear relationship between the total number of receptors and the observed rate constant for the accumulation of cAMP. They suggested that the generation of the response (cAMP accumulation) was dependent on the ß-AR mediated activation of an effector. In the rabbit aorta we similarly demonstrate that both the observed rate constant and delay time for the onset of the ß-adrenergic desensitization shows saturable dependence on agonist concentration. However, *the agonist* in this study mediates the contractile response while *the response* that is measured is the onset of the desensitization of the ß-AR. Thus, a mode of interaction between the stimulus for contraction and the

molecular events that lead to ß-AR desensitization needs to be elucidated.

In the rabbit aorta, activation of the α_1-AR or $5HT_2$ receptor leads to turnover of polyphosphoinositides and eventually causes the activation of PKC by the generation of diacylglycerol and elevation of Ca^{2+} levels in the cell. Because the t_d and k_{des} depend on the occupancy of these PI-linked receptors (fig. 4) and do not depend on the occupancy of the ß-AR we propose that the rate limiting step for desensitization of the ß-AR may be a PKC-mediated phosphorylation of the ß-AR.

There is considerable support in the literature for the hypothesis that the ß-AR desensitization is mediated via a phosphorylation event. The ß-AR has been shown to be phosphorylated by at least 3 distinct kinases: ß-ARK (Benovic et al., 1986), cAMP-dependent protein kinase (PKA; Benovic et al., 1985; Bouvier et al., 1987), and protein kinase C (PKC; Bouvier et al., 1987). However, the is a continuing search for the identity of the kinase(s) responsible for desensitization *in vivo* did not yet provide an unequivocal answer. There is little doubt that ß-ARK can specifically phosphorylate the agonist-occupied ß-AR as well as several other receptors coupled to adenylyl cyclase (Benovic et al., 1986; Bouvier et al., 1988). However, other studies also suggest important roles for PKA

(Sibley et al., 1984; Clark et al., 1988) and PKC (Yamashita et al., 1987; Toews et al., 1987; Hui and Yu ,1989) in agonist-induced ß-AR regulation. The findings reported here are consistent with a role for PKC in the desensitization response.

The reports in the literature that are most relevant to the current work have focused on the contributions of the various kinases to the modulation of responses in which more than one effector pathway is activated simultaneously (e.g. PI turnover as well as adenylyl cyclase). Toews et al. (1987) showed that the combination of PMA (an activator or PKC) and ISO leads to a greater desensitization than either agent alone in a human astrocytoma cell line. Yamashita et al. (1987) confirmed that PMA induced desensitization is enhanced by ISO activation of the ß-AR. These effects of PMA are particularly relevant in consideration of the work presented here because the response to ISO is always visualized in aortic rings that have been precontracted either with PE or 5HT and are consistent with the hypothesis that the desensitization in this system may be mediated in part by PKC.

In summary, the relaxant effect of ISO in precontracted rabbit aorta depends on both the identity and the concentration of the agonist eliciting contraction. The nature of this interaction must be examined in further detail to elucidate the molecular steps in the mechanism of functional interactions.

However, we can conclude at this stage that such functional interactions may be additive based on the inverse dependence of the k_{rel} on the occupancy of the α_1-AR or $5HT_2$ receptor. Furthermore, the dependence of the rate constant for desensitization on the concentration of contractile agonist suggests an important role for PKC-mediated phosphorylation of the ß-AR in the process of desensitization.

Figures for paper II

Table I

Characteristics of the responses to ISO in tissues precontracted with either 1 μM PE or 1 μM 5HT. The summary of results describing the relaxation and desensitization responses in the presence of PE was adapted from Keitz et al., paper I.

Table I: Characteristics of relaxation and desensitization

Response	*Steady-State Parameters*	*Kinetic Parameters*
Relaxation in the presence of 5HT	**R/C** (fractional relaxation) shows a saturable dependence on [ISO]. EC_{50} = 0.062 μM; E_{max} = 0.716; slope index = 0.96 (n = 6 rings from 3 rabbits)	k_{rel} (observed rate constant for relaxation) shows a saturable dependence on [ISO]. EC_{50} = 0.017 μM; E_{max} = 0.044 sec^{-1}; slope index = 1.23 (n = 6 rings from 3 rabbits)
Desensitization in the presence of 5HT	**D/R** (fractional desensitization) shows minimal change over [ISO] (from 0.1 to 10 μM). avg. **D/R** = 0.77± 0.06 (n = 16 repetitions from 3 rabbits)	k_{des} (observed rate constant for desensitization) shows minimal change over [ISO] (from 0.03 to 10 μM). avg. k_{des} = $(8.1\pm0.6)*10^3$ (n = 23 repetitions from 3 rabbits) **td** shows no dependence on [ISO]. avg. **td** = 65 ± 13 secs (n = 22 repetitions from 3 rabbits)
Relaxation in the presence of PE	**R/C** (fractional relaxation) shows a saturable dependence on [ISO]. EC_{50} = 0.07 μM; E_{max} = 0.8; slope index = 1.26	k_{rel} (observed rate constant for relaxation) shows a saturable dependence on [ISO]. EC_{50} = 0.023 μM; E_{max} = 0.046 sec^{-1}; slope index = 1.01
Desensitization in the presence of PE	**D/R** (fractional desensitization) shows minimal change over [ISO] (from 0.1 to 10 μM). avg. **D/R** = 0.83± 0.02	k_{des} (observed rate constant for desensitization) shows minimal change over [ISO] (from 0.03 to 10 μM). avg. k_{des} = $(4.7\pm0.2)*10^3$ **td** shows no dependence on [ISO]. avg. **td** = 191 ± 6 secs

Figure 1:

Representative ISO induced responses in paired aortic rings from the same animal. Tissues were contracted to the same level of tone (~6-7 grams) by 1 μM PE, 10 μM 5HT, 30 μM HA or 40 mM KCl. 1μM ISO was added in each tracing at the dot. Trials using HA were performed in the presence of 1μM tiotidine to block HA action on the H_2 receptor.

Figure 1

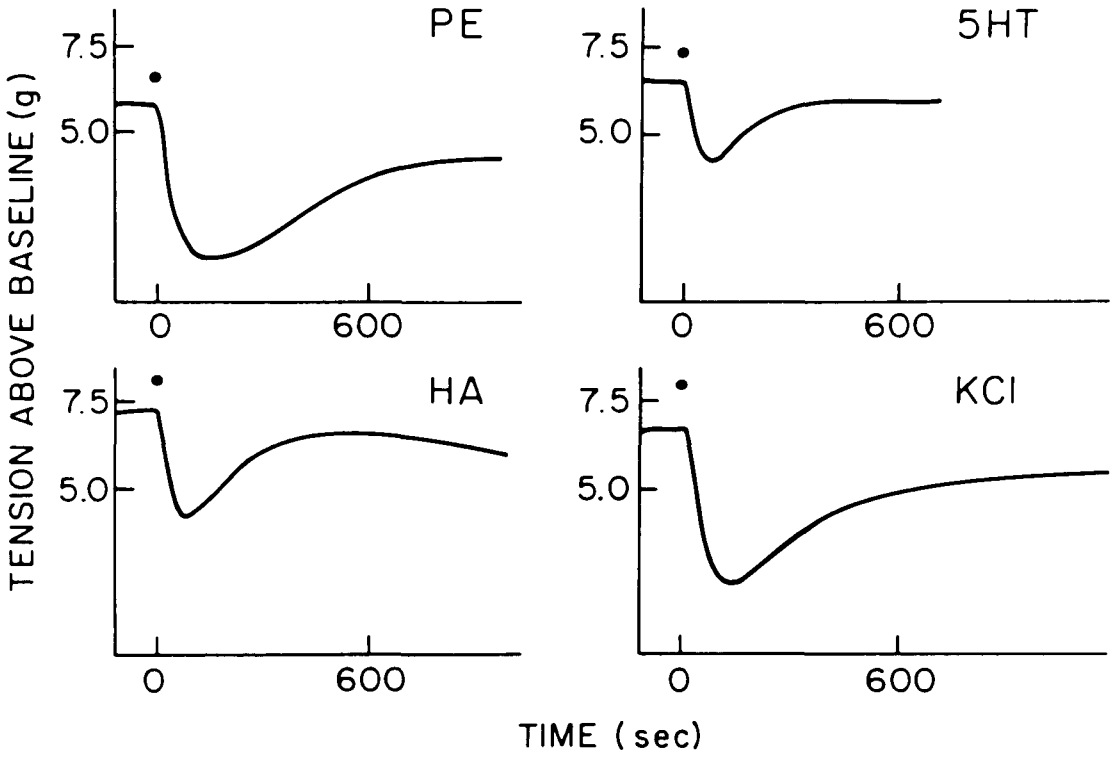
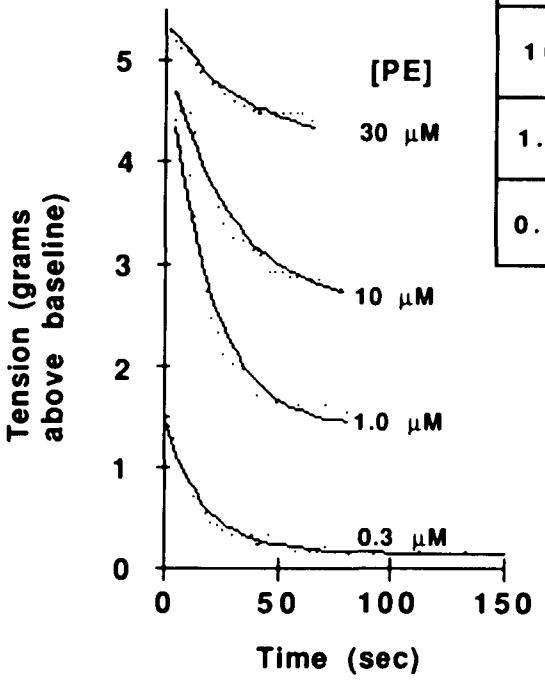


Figure 2:

The effect of changing PE concentration on the relaxation response to 0.3 μM ISO in a single ring of rabbit aorta. The data points were collected by computer and fitted to equation 3 ($r^2 \geq 0.98$) for the estimation of the parameters **R** and $\mathbf{k_{rel}}$ (Methods). The table inset gives the values for the measured contraction (**C**), the fitted parameter $\mathbf{k_{rel}}$ and the fractional relaxation (**R / C**) for the four curves shown. The desensitization response for each curve is not shown.

Figure 2



[PE] (μM)	fraction of KA	C (g)	krel (sec -1)	R/C
30	40.5	5.30	0.028	0.215
10	13.5	4.70	0.036	0.451
1.0	1.35	4.35	0.052	0.680
0.3	0.405	1.54	0.056	0.896

Figure 3

The dependence of the fractional relaxation (**R/C**) on the concentration of PE or 5HT. Concentrations of contractile agonists are represented as [drug]/K_A. The ß-Adrenergic responses were elicited by the addition of ISO (0.3 μM) to precontracted rings of rabbit aorta. The open squares are responses in the presence of PE and the closed diamonds are responses in the presence of 5HT. The points represent mean fractional responses ± S.E.M. of the observed data from (n) repetitions in aortic rings taken from 6 rabbits in the presence of PE and 3 rabbits in the presence of 5HT. The (n) for the closed diamonds are reported above the points, and the (n) for the open squares below the points. **R/C** in the presence of 5HT was assayed in the presence of BHC (15 μM) to block 5HT's action on the α_1-AR.

Figure 3

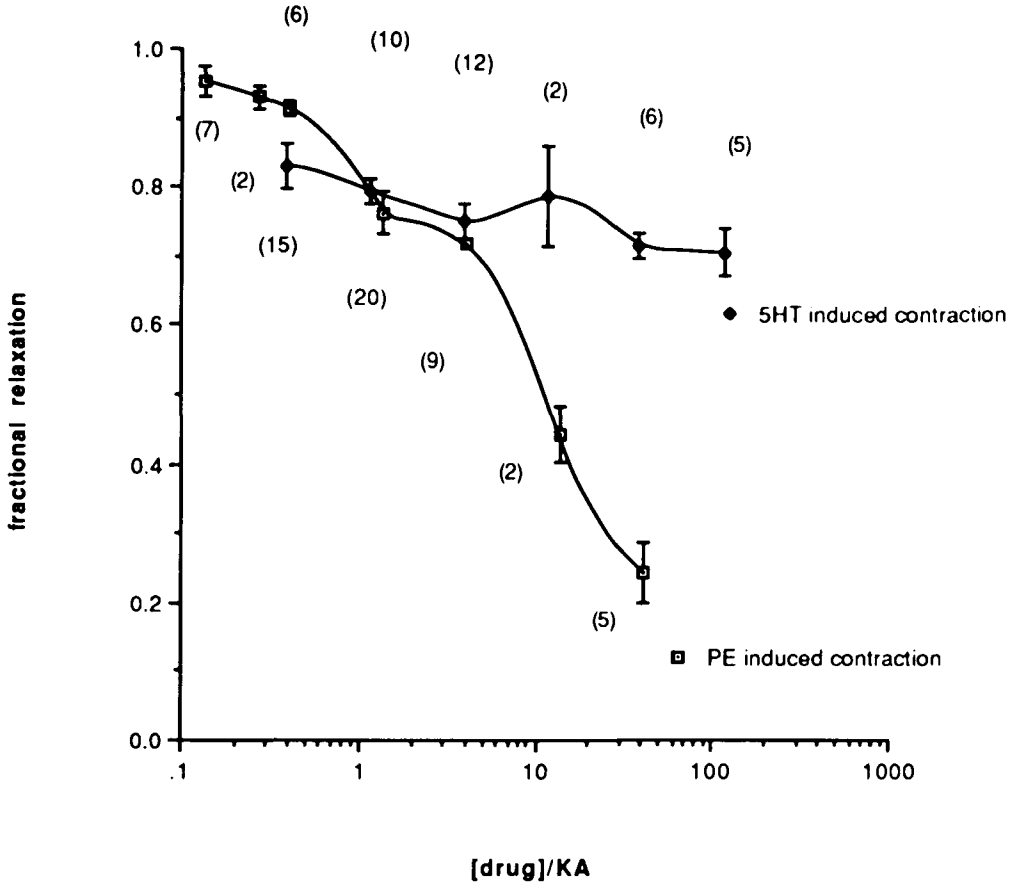

(6)
(10)
(12)
(2)
(6)
(5)
(7)
(2)
(15)
(20)
(9)
(2)
(5)
1.0
0.8
0.6
0.4
0.2
0.0
.1
1
10
100
1000
fractional relaxation
[drug]/KA
5HT induced contraction
PE induced contraction

Table II

A representative subset of data illustrating the relationship between $\mathbf{k_{rel}}$ and fractional occupancy ([drug]/K_A). Kinetic parameters were estimated from fitting the response curves to eq. 1 or eq. 3 (Methods). For all fits $r^2 \geq .98$. n represents the number of rings included in the representative ANOVAR.

Table II

Relationship between krel and [drug]/K_A

[drug] (μM)	*[drug]/K_A*	*n*	*Mean k_{rel} ± SEM (sec^{-1})*
PE*			
0.3	0.405	8	0.049 ± 0.004
1.0	1.35	8	0.046 ± 0.005
10	13.5	8	0.044 ± 0.006
5HT			
0.1	0.385	8	0.056 ± 0.007
0.3	1.16	8	0.047 ± 0.006
1.0	3.85	8	0.042 ± 0.005
10	38.5	8	0.038 ± 0.006

*ANOVAR showed significant effects of changing concentration in each individual ring ($p=0.0003$)

**ANOVAR showed significant effects of changing concentration in each individual ring ($p=0.0001$)

Figure 4

The dependence of the rate constant for desensitization (k_{des}) on the fraction of receptors occupied by PE or 5HT. All responses are to 0.3 μM ISO. The open squares are responses to ISO in the presence of PE and the closed diamonds are responses in the presence of 5HT. The points represent mean fractional responses ± S.E.M. of the observed data from (n) repetitions in aortic rings taken from 3 rabbits. The parameters of the CRC in the presence of PE are E_{max} = 0.0044 ± .0004, EC_{50} = 0.43 μM ± 0.09 μM and n= 1.3 ± 0.36. The parameters of the CRC in the presence of 5HT are E_{max} = 0.0050 ± .0005, EC_{50} = 0.08 μM ± 0.03 μM and n= 0.93 ± 0.5. For both curves $r^2 > 0.99$.

Figure 4

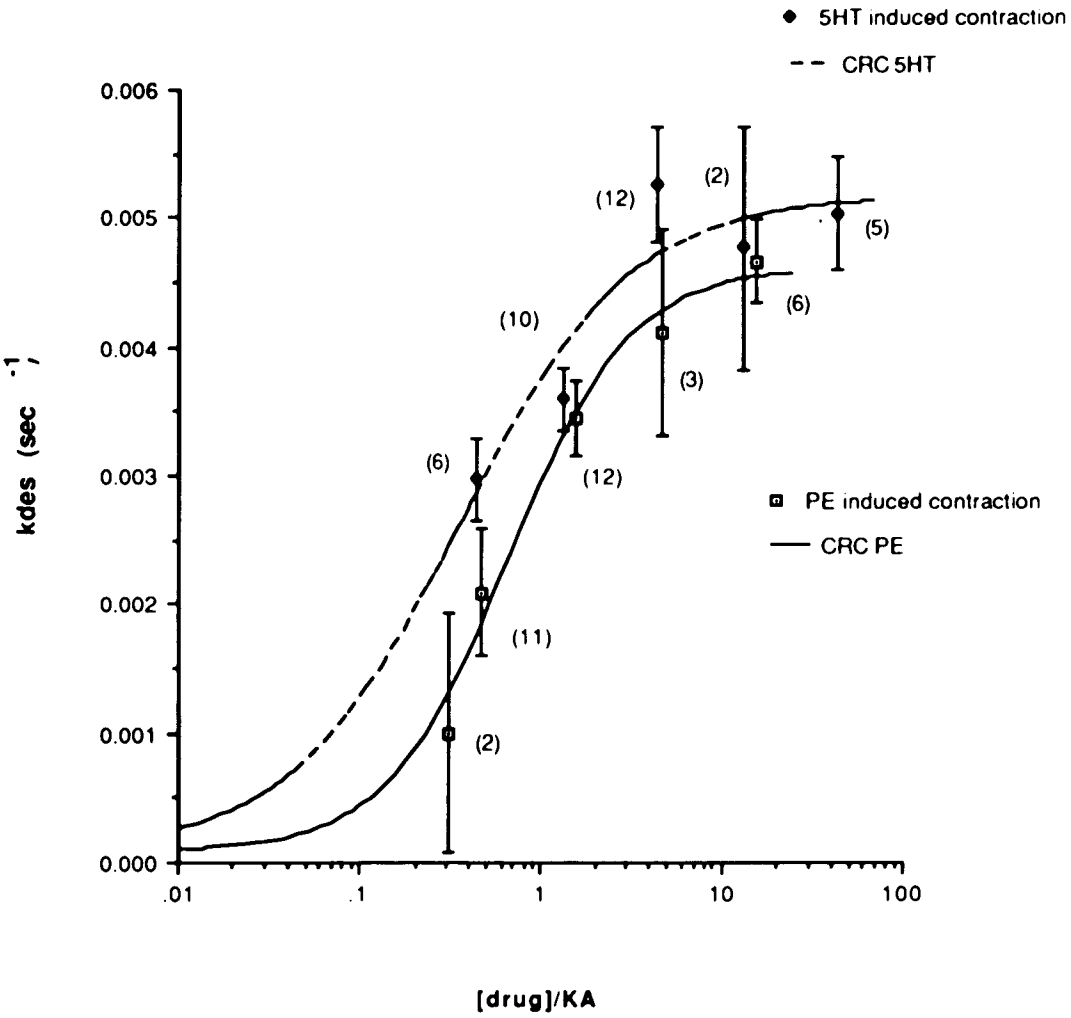

5HT induced contraction
CRC 5HT
PE induced contraction
CRC PE
kdes (sec -1)
0.006
0.005
0.004
0.003
0.002
0.001
0.000
.01
.1
1
10
100
[drug]/KA
(12)
(2)
(5)
(10)
(6)
(3)
(6)
(12)
(11)
(2)

Figure 5

The dependence of t_d on the concentration of PE (open squares) or 5HT (closed diamonds). Concentrations are expressed as [drug]/K_A. Responses were to 0.3 μM ISO. Values are the mean td ±S.E.M. Values were taken from (n) repetitions from aortic rings of 6 rabbits. The (n) for the closed diamonds is reported below each point, and the (n) for the open squares is above each point.

Figure 5

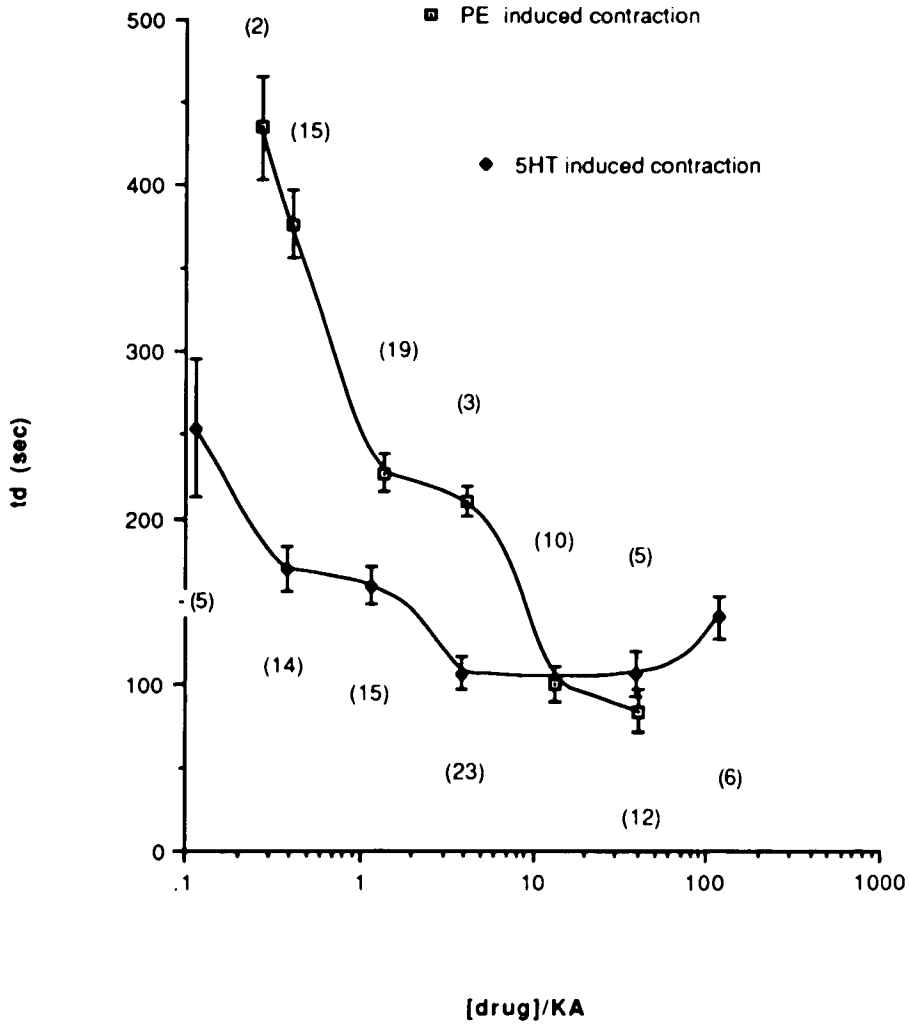

PE induced contraction
5HT induced contraction
500
400
300
200
100
0
.1
1
10
100
1000
(2)
(15)
(19)
(3)
(10)
(5)
(5)
(14)
(15)
(23)
(6)
(12)
td (sec)
[drug]/KA

Figure 6

Scheme of functional interaction: a possible interaction between the chain of stimuli produced by ISO's activation of the ß-AR and PE's activation of the α-AR. Activation of each receptor leads to a chain of stimuli that interact at some point. In this example, the stimuli for relaxation and contraction are additive resulting in one single "final" stimulus that is responsible for the final observed tissue tone. The functional relationship between the "final" stimulus and the observed tone is hyperbolic and the slope factor of this curve represents the k_{rel}. (adapted from Mackay, 1981).

Figure 6

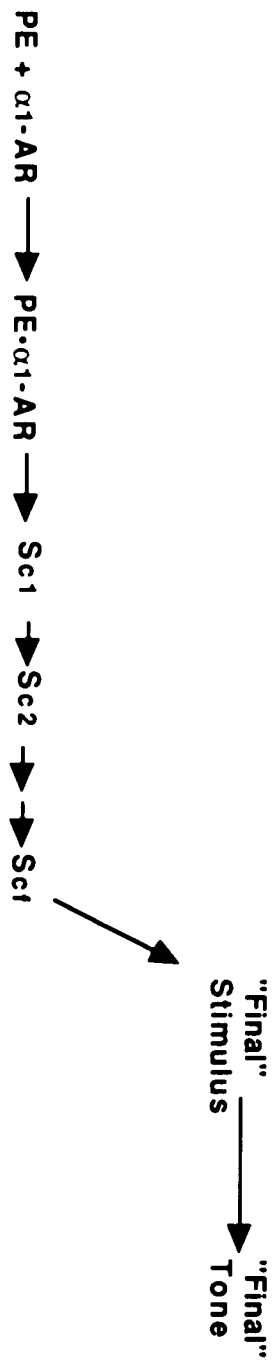
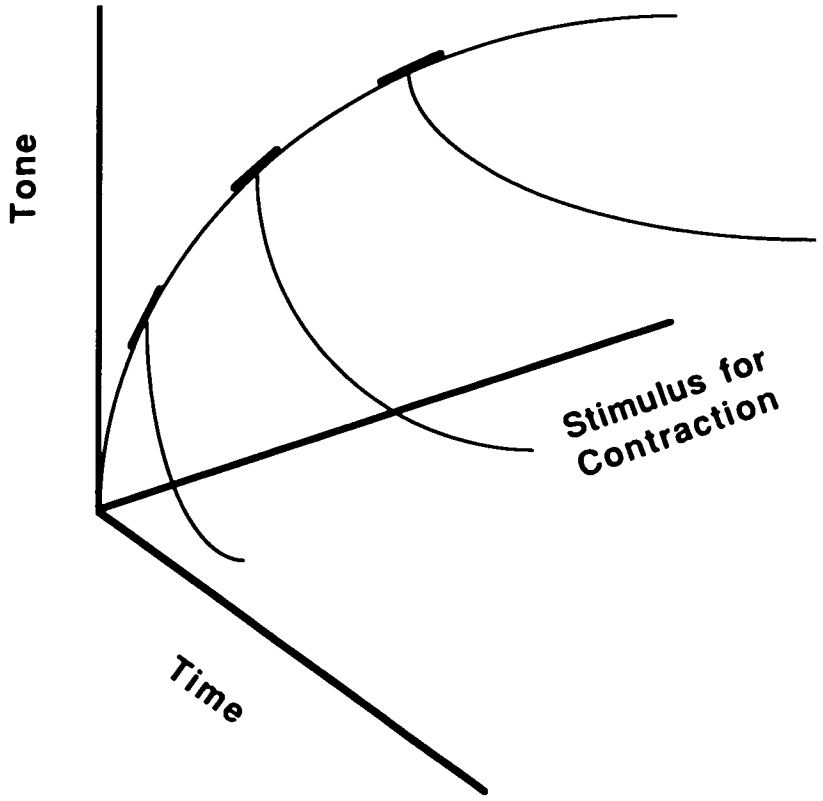

ISO + β2-AR
ISO·β2-AR
Sr1
Sr2
Srf
PE + α1-AR
PE·α1-AR
Sc1
Sc2
Scf
"Final" Stimulus
"Final" Tone

Figure 7

Three dimensional plot of functional interactions in the rabbit aorta model: a schematic diagram illustrating three of the four variables that describe the functional interactions in this intact tissue. The axis depict the following variables: 1- the stimulus for contraction; 2- tissue tone; and 3- time. The stimulus for relaxation is not shown as it remained constant throughout the experiments.

Figure 7



paper III

Functional interactions in Smooth Muscle: The effects of altering contractile efficacy on ISO-induced relaxation and desensitization

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Functional interactions can be probed in isolated tissues by simultaneous activation of distinct membrane receptors which interact in either an antagonistic or synergistic manner. In the rabbit aorta such interactions can be visualized as time dependent changes in muscle tone. In this system a contraction produced by phenylephrine (PE) or serotonin (5HT) can be functionally antagonized by isoproterenol (ISO) acting on the ß-AR (Keitz et al., papers I and II). We have previously reported that this functional antagonism is accompanied by a desensitization that is visualized as a regaining of tension. This biphasic response to ISO (i.e., relaxation and desensitization) was quantitated using an exploratory kinetic model which separated the relaxation and desensitization responses (Keitz et al., paper I).

In previous work we examined the dependence of the ß-adrenergic relaxation and desensitization on the concentration of ISO in the presence of a fixed concentration of PE or 5HT (Keitz et al., paper I). We also quantitated the dependence of the responses mediated by activation of the ß-AR by 0.3 µM ISO on the concentration of contractile agent (either PE or 5HT; Keitz et al., paper II). The relaxation response parameters, fractional relaxation and k_{rel}, were dependent on the concentration of ISO in a saturable manner and inversely

related to the concentration of PE or 5HT. However, the relaxant effect of ISO was considerably greater in rings contracted with high concentrations of 5HT than in rings contracted with high concentrations of PE (Keitz et al., paper II). One possible explanation that was presented (Keitz et al., paper II) for the difference in the dependence of the fractional relaxation on PE vs 5HT was that ISO differentially alters the efficacy of the systems. Thus, both agonists became less efficacious, but PE retained its characteristics as a full agonist while 5HT appeared as a partial agonist. Because it is difficult to compare efficacies of responses that are mediated through the activation of different membrane receptors, *we chose to study the dependence of the fractional relaxation on changes in efficacy in a single receptor system.*

The desensitization parameters, $\mathbf{k_{des}}$ and $\mathbf{t_d}$, were found to be independent of the concentration of ISO (Keitz et al., paper I) but depended on the concentration of contractile agonist in a saturable manner (Keitz et al., paper II). This suggested that the desensitization of ß-AR depended on the activation of the α_1-AR or $5HT_2$ receptor and the production of a stimulus for contraction. Therefore, *another goal of this work was to characterize the effects of changes in contractile efficacy on the desensitization response.*

The concept of efficacy: Stephenson (1956) introduced the concept of *efficacy* as the capacity of a drug to initiate a response. Stephenson made an attempt to define the relationship between *stimulus*, which was proportional to occupancy, and pharmacological response. The relationship, that was expressed as 'equal stimuli yield equal responses', leads logically to the conclusion that drugs with different efficacies need to occupy different fractions of the total number of receptors to produce equal stimuli. The condition of 'equal stimuli produce equal responses' (the 'null' condition) leads only to the definition of a relative scale of efficacies rather than to an absolute scale. The relative scale of efficacies is a direct result from the experimental conditions which require that measurements of <u>two</u> agonists with different efficacies will be performed on the same system to satisfy the null condition. The null condition does not allow comparisons of responses elicited by the activation of different receptors (eg. α_1-AR or $5HT_2$ receptor). These inadequacies stem from the lack of a chemical identity for the concept of efficacy and from the undefined relationship between the stimulus and the effect.

The operational model of Black and Leff (1983) demonstrated that a hyperbolic relationship between stimulus and effect is a necessary outcome from the observed

hyperbolic nature of the relationship between the pharmacological effect and the concentration of the agonist. In the relatively recent formulation of the *operational model of agonist action* by Black and Leff efficacy has been identified with a parameter, K_E, that relates to a specific mechanism: the transduction of the agonist-receptor complex, AR, into a pharmacological effect. Efficacy, named in this model the 'transducer ratio', assumed the form: $\tau=[R_0]/K_E$, where $[R_0]$ is the total number of receptors and K_E is defined operationally as the concentration of the agonist-receptor complex that yields half-maximal response. Black's K_E has a clear meaning in well understood responsive systems such as the activation of adenylate cyclase by β-adrenergic agonists (DeLean et al., 1978). In such systems K_E becomes the affinity of the agonist-receptor complex for the effector and thus acquires a well defined chemical identity. Furthermore, the efficacy term, τ, is related to the maximal response (α; i.e. assymptote) by the following equation: $\tau=\alpha/(1-\alpha)$. However, it is unclear whether τ, the transducer ratio can be used to compare responses that were elicited by activation of different receptor systems.

The concept of efficacy in an intact tissue preparation: In an intact tissue preparation the pharmacologic effect is measured as a function of the concentration of the agonist.

Detailed models have been constructed to describe the occupancy of the receptor and the activation of the effector by the drug•receptor complex as the first consequence of occupancy of the receptor by an agonist. However, much less is known about the translation of that initial signal into an integrated response in a tissue preparation. This is because in many cases only a portion of the events that occur following receptor activation are known. Thus, the transducer serves of as a 'black box' that comprises all the steps, known and unknown, which translate receptor activation into the observed response.

Fortunately, detailed knowledge of the steps that relate stimulus to response is not necessary for the quantitation of drug effects. For drugs acting through the same receptor system, the null condition can be used to compare equal states thereby cancelling the effects of the "unknown" steps by assuming that they are equal. Furthermore, the efficacy of the transduction can be controlled either at early or late stages within the black box. For example, a series of partial agonists with varying efficacies represent changes at an early point of translation of occupancy to stimulus. On the other hand, calcium channel active drugs alter a later event in the production of contractile response by altering the Ca^{2+} fluxes and the resulting Ca^{2+} concentration in the cell. Thus, *the*

final goal of this work was to study the dependence of the ß-adrenergic relaxation and desensitization response parameters on the contractile efficacy by varying efficacy in two ways that address very different points along the pathway from receptor occupancy to final response. In one set of experiments, the availability of a large number of $5HT_2$ agonists of varying efficacy allowed the study of the response to ISO in the presence of equal occupancies of a series of $5HT_2$ agonists (Stollak and Furchgott, 1983; Clancy and Maayani, 1985; Cory et al., 1986). In another set of experiments, the contractile efficacy was altered by the addition of the calcium channel activator, Bay K 8644 (Bay K) and the calcium channel inhibitor, nifedipine (NIF).

METHODS.

Tissue preparation.

Male New Zealand White Rabbits weighing between 1.4-1.8 kg. (Ace Animals, Inc., Boyertown PA) were sacrificed by CO_2 asphyxiation and the thoracic aortae were rapidly excised and prepared as previously described (Keitz et al., paper I). The tissues were cut into 4-6 rings 0.5-0.6 cm in width which were suspended between stainless steel hooks in 20-ml organ baths. The baths contained Krebs-bicarbonate buffer bubbled with 95% O_2-5% CO_2 to maintain a pH of 7.4 ± 0.2 at 36° ± 1 °C. The tissues were initially set to ~2 g tension and allowed to relax until a stable tension was maintained. The minimum stabilization time was 1 hour. Once resting tension was stabilized, baseline tension was set to a force between 1 and 2 g. All rings were primed twice by eliciting a contraction with 10 μM PE. Drugs were removed from the bath by draining and replacing the buffer.

Solutions.

Krebs-bicarbonate buffer contained the following components (in mM) in glass distilled water: NaCl, 110; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 2.35; KH_2PO_4, 1.2; $NaHCO_3$, 25; glucose, 11; Na_2EDTA, 0.03.

Chemicals.

The following drugs were used (abbreviations): N,N dimethyl 5-hydroxytryptamine (Bufotenin; BUF) and N,N dimethyl tryptamine (DMT); [NIDA]; 2-(1-piperazinyl) quinoline (quipazine; QUIP) [Miles Laboratories Inc., Elkhart, Ind.]; Benextramine tetrahydrochloride (BHC) [Aldrich Chemical Co., Milwaukee, WI]; (-)Isoproterenol phosphate; (ISO) [Sigma, St. Louis, MO]; All other chemicals were of analytical grade as previously reported (Clancy and Maayani, 1985).

Responses to ISO in rings precontracted with a series of $5HT_2$ agonists with varying efficacy.

Each aortic ring was contracted by a saturating concentration of one of the $5HT_2$ agonists (10 μM 5HT and 20 μM each of DMT, BUF, QUIP). In these precontracted rings, a relaxation response was induced by 0.3 μM ISO. Figure 1 shows a typical series of ISO responses on tissues precontracted with various $5HT_2$ agonists. At the end of the desensitization in each trial, when tissue tension reached steady state, the tissue was washed three times and left to rest for one hour to allow for recovery of responsiveness. All trials were conducted after exposure to 15 μM benextramine tetrahydrochloride (BHC) for 30 minutes to

block agonist action on the α-adrenergic receptor (α-AR) . Blockade of the α-AR was confirmed by lack of responsiveness to 10 μM PE.

Although all drugs were tested in every ring, the response to DMT was too small in rings from three of the six animals tested to allow a reliable estimation of the ß-adrenergic response parameters. For each agonist, the intrinsic activity was determined relative to the response to 5HT in the same ring. In most rings, responses to ISO in the presence of 5HT were tested twice to assure that the tissue was still viable and that the partial agonists were adequately washed from the tissue. In these rings, the first trial in the presence of 5HT was always used for determination of intrinsic activity.

Responses to ISO in rings precontracted with PE or 5HT: the effect of calcium channel active drugs.

A control trial consisted of eliciting a contraction by PE or 5HT and the subsequent addition of 0.3 μM ISO to induce relaxation and desensitization. The tissue was then washed three times in drug free buffer for 30 minutes and incubated for an additional 30 minutes with either 1 μM NIF or 0.3 μM Bay K before repeating the trial in the presence of NIF or Bay K. The concentration of PE or 5HT was 1 μM in the

trials testing the effects of NIF. The concentration of PE was 0.1 μM in the trials testing the effects of Bay K while the concentration of 5HT was 0.06 μM in these trials. Because NIF and Bay K were not easily washed from the tissue, each ring was exposed only to one of the dihydropyridines (DHP) and was not used for further testing. Thus, each ring served as its own control. Parameters were compared using a paired t-test with $p<0.05$ considered significant.

Statistics.

For those experiments in which the series of $5HT_2$ agonists were tested, response parameters from consecutive trials on the same aortic rings were compared. One way Analysis of Variance (ANOVA) was performed before multiple comparisons were done to compare the different groups. Multiple comparisons were made using paired t-tests with the significance level adjusted with the Bonferroni correction (Krauth, 1988).

For those experiments in which responses to ISO were tested in rings contracted with PE or 5HT in the presence and absence of the calcium channel active drugs, paired samples were compared using paired t-tests. A value of $p<0.05$ was considered significant.

Collection of data points.

Isometric contractions were measured with Grass FT03C force displacement transducers connected to the Grass polygraph model 7D. Data points corresponding to tension were collected at a frequency of 0.04 - 1 Hz and stored on floppy disk for subsequent analysis as was previously described (Keitz et al., paper I). Digital values of muscle tension were transferred to a Sun 3/110 workstation for non-linear analyses with the PROPHET II computer program.

The kinetic model:

The relaxation and desensitization responses to ISO were separated using a model previously described (Keitz et al., paper I). The relaxation response was modeled as an exponential decay:

$$T = C - R\,(1 - e^{(-k_{rel} * t)}) \qquad (1)$$

where **T** is the observed tissue tension, **C**-the initial contraction, **R** the magnitude of the ISO induced relaxation, k_{rel}-the observed rate constant for relaxation and t- the time elapsed since the addition of ISO. **C** was measured from the experimental curve immediately before the addition of ISO to the bath. The values for **R,** and k_{rel} were estimated by

fitting experimental data to this model with a non-linear regression procedure.

The desensitization response was also modeled as a simple exponential increase in tension:

$$T = P + D(1- e^{(-k_{des} * (t-t_d))}) \qquad (2)$$

where **T** is the observed tissue tension, **P** is the plateau from which the regaining of tension begins, **D** is the magnitude of the ISO induced desensitization, k_{des} is the observed rate constant for desensitization and t_d is time following the addition of ISO before desensitization is observed. The valid domain of equation 3 is only for $t \geq t_d$. The values of **D**, k_{des}, and t_d were estimated using a nonlinear fitting procedure whereas the plateau, **P**, was measured.

When t_d approaches zero (i.e. when the desensitization response begins before the relaxation response has reached its maximum) the entire tracing must be described by a single equation in which **P** is replaced by eq.1.

$$T= C - R (1-e^{(-k_{rel} * t)}) + D (1-e^{(-k_{des} * t)}) \qquad (3)$$

All of the data were evaluated initially with Eq. 1 and 2. When t_d was $\leq$ 5 times the half-life for relaxation ($T_{1/2} = \ln 2/k_{rel}$), all the parameters were recalculated using equation 3 and these values were used for subsequent analysis. In the fitting, td was constrained to be ≥ 0 and **R** was constrained to

be ≤ **C**. In order to compare different rings, the extent of relaxation, is described as fractional relaxation, **R/C**. Likewise, the desensitization is represented as fractional desensitization, **D/R**.

RESULTS.

DMT, BUF, QUIP and 5HT contract the rabbit aorta via activation of $5HT_2$ receptor (Stollak and Furchgott, 1983; Clancy and Maayani, 1985; Cory et al., 1986). The intrinsic activities (α) have been determined for these agonists relative to 5HT: α_{DMT} = 0.15±0.02 (n=5 rings from 3 rabbits), α_{BUF} = 0.46 ±0.04 (n=16 rings from 6 rabbits), and α_{QUIP} = 0.69±0.03; (n=16 repetitions from 6 rabbits). These intrinsic activities are similar to those previously determined in the rabbit aorta preparation (Cory et al., 1986; Clancy, 1987).

Characterization of the relaxation response to ISO in tissue precontracted with agonists of varying intrinsic activity. A representative example of the action of ISO in a rabbit aortic ring contracted with saturating concentrations of DMT, BUF, QUIP and 5HT is illustrated in Fig. 1. The biphasic response, seen clearly in each of the tracings, is composed of an initial relaxation followed by a subsequent regaining of tension.

The mean values of the fractional relaxation induced by 0.3 μM ISO in tissues contracted with these four agonists are shown in Fig. 2A. Clearly, the extent of the fractional relaxation depends on the intrinsic activity (α) of the contractile agonist. ISO is able to relax the tissue to baseline

tension when the tissue is contracted with DMT, the agonist with the lowest α. As the intrinsic activity of the contractile agonist increases, the fractional relaxation decreases to a value of approximately 0.6 for 5HT, the agonist with the highest α. Results from rings for which data were available for all four agonists showed a significant rank order of relaxation that depends on efficacy. The only significant rank order of fractional relaxation from the complete data was that QUIP and 5HT relaxed less than DMT and BUF (ANOVA, $p<0.0001$; t-tests with Bonferronie correction $p<0.05$). Paired analysis for those rings in which data were available for BUF, QUIP and 5HT (n=16) showed that the rank order of fractional relaxation is QUIP< 5HT< BUF ($p<0.05$ with bonferronie correction).

The rate constant for ISO induced relaxation, $\mathbf{k_{rel}}$, showed no apparent dependence on the intrinsic activity of the contractile agonist (Fig. 2B). Average values for $\mathbf{k_{rel}}$ in the presence of saturating concentrations of the contractile agent are: DMT: 0.037 ±0.009 sec^{-1} (n=5 rings from 3 rabbits); BUF: 0.051 ±0.004 sec^{-1} (n=17 rings from 6 rabbits); QUIP: 0.034± 0.003 sec^{-1} (n=16 rings from 6 rabbits); and 5HT: 0.044± 0.002 sec^{-1} (n=32 rings from 6 rabbits) respectively (ANOVA $p>0.05$). Results from rings in which both BUF and 5HT were tested (n=17) showed a significant difference between 5HT and BUF (paired t-test $p<0.05$).

Characterization of the desensitization response to ISO in tissue precontracted with agonists of varying efficacy. Fractional desensitization in the presence of agonists of varying intrinsic activity is shown in Fig. 3A. The results from experiments in which all four agonists were tested on the same ring indicate that the fractional desensitization depends on the efficacy of the drug (ANOVAR, $p<0.05$). The fractional desensitization in the presence of a saturating concentration of 5HT is ~0.8. This value is similar to the values for fractional desensitization previously observed (see Keitz et al., paper I and II). At the low range of intrinsic activities the average fractional desensitization induced by ISO is greater than 1. QUIP shows an unusual behavior; in spite of its lower efficacy, a saturating concentration of QUIP leads to a fractional desensitization of ~0.6 of the ISO response. The observed rank order of the fractional desensitization is DMT~BUF>5HT>QUIP($p<0.05$).

The observed rate constant for desensitization, $\mathbf{k_{des}}$, also depends on the intrinsic activity of the agonist eliciting contraction (Fig. 3B; (ANOVAR, $p<.02$). Results from experiments in which all drugs were tested show that the values for $\mathbf{k_{des}}$, in the presence of DMT and BUF were significantly smaller than those in the presence of QUIP and 5HT ($p<0.05$). Furthermore, those rings in which both BUF and

5HT were tested (n=16) k_{des} was significantly larger in the presence of 5HT than in the presence of BUF (paired t-test $p<0.05$). The observed rank order of k_{des} is DMT≤BUF<QUIP<5HT.

Results from experiments in which all drugs were tested on the same aortic ring suggest that there was a significant dependence of t_d on the intrinsic activity of the drug (ANOVAR $p<0.02$). The average values shown in Fig. 3C suggest that the t_d in the presence of DMT (236±50,n=5 rings), BUF (217 ± 16, n=17 rings) and QUIP (221± 18, n=16 rings) were similar while t_d in rings contracted with 5HT (111±10, n=32 rings) was significantly smaller ($p<0.05$).

Effects of NIF on the response to ISO in tissue precontracted with PE or 5HT. Table I summarizes the effect of NIF on the parameters that describe contraction elicited by either PE or 5HT and the relaxation and desensitization induced by 0.3 μM ISO. Qualitatively the effect of NIF on the parameters that describe the contraction and relaxation processes is the same regardless of whether the contraction was elicited by PE or by 5HT. However, there was no consistent effect of NIF on the desensitization parameters in rings contracted with PE or 5HT. Representative curves showing the effects of NIF on the response to ISO in tissue precontracted with PE are shown in Fig. 4. The data points were fitted to equations 1 and 2.

The mean contraction elicited by either 1 μM PE or 1 μM 5HT was decreased by NIF. The mean contraction induced by PE in the presence of 1 μM NIF was 48% of the control contraction. The addition of NIF also significantly reduced the mean contraction to 1μM 5HT, however in this case the mean contraction for the control in the presence of NIF was 67% of the control contraction.

In addition, the ISO induced responses were affected by NIF. Thus, the fractional relaxation (**R/C**) and the rate constant for relaxation were increased by NIF. The fractional desensitization in the presence of 1μM PE or 1μM 5HT was decreased significantly. The rate constant for desensitization was also decreased by NIF in rings precontracted with PE but not in rings precontracted with 5HT. Similarly, the t_d was significantly increased by NIF in rings precontracted with PE but not in those precontracted with 5HT.

Effects of Bay K on the response to ISO in tissue precontracted with PE or 5HT. Table II summarizes the effect of Bay K on the parameters that describe contraction elicited by either 0.1 μM PE or 0.06 μM 5HT. The effect of Bay K on the relaxation and desensitization induced by 0.3 μM ISO is also shown. The effect of Bay K on the parameters that describe the contraction and relaxation processes is qualitatively the same regardless of whether the contraction

was elicited by PE or 5HT. However, the effects of Bay K on the desensitization parameters in rings precontracted with PE or by 5HT were not consistent. Representative curves showing the effects of 0.3 μM Bay K on the response to ISO in tissue precontracted with PE are shown in Fig. 5. The data points were fitted to equations 1 and 2.

The addition of 0.3 μM Bay K significantly increased the mean contraction to 0.1 μM PE and to 0.03 μM 5HT. For rings contracted with either PE or 5HT, the fractional relaxation (**R/C**) was not changed while the rate constant for relaxation was significantly decreased. Bay K (0.3 μM) significantly decreased the fractional desensitization in the presence of 0.1 μM PE from complete desensitization to ~0.8 but not in the presence of 5HT. In rings precontracted with either PE or 5HT, there was no significant effect of Bay K on $\mathbf{k_{des}}$ or $\mathbf{t_d}$.

DISCUSSION

The generation of a receptor-mediated response is governed by two fundamental properties of the drug-receptor interaction: agonist affinity and efficacy. Agonist affinity describes the tightness of drug binding to the receptor whereas agonist efficacy represents the efficiency of the D•R complex in activating the effector mechanism to produce the final response. In a chemical representation, efficacy can be considered the affinity of the D•R complex to a transducer or an effector (Black and Leff, 1983). In such a formulation the agonist affinity and efficacy define the relationship between drug concentration and response. Furthermore, because of their similar chemical nature the same response level can be achieved either by a change in the concentration of the agonist which alters the occupancy of the receptor, or by a change in efficacy, e.g. by the use of a partial agonist.

We have shown that a contraction of the rabbit aorta elicited by PE or 5HT can be relaxed by the addition of ISO and that this relaxation is followed by a desensitization of the relaxing response (Keitz et al., paper I). The relaxation response is dependent upon the concentration and the identity of contractile agonist (Keitz et al., paper II). We have also shown that the ISO response parameters in precontracted rabbit aortic strips depend on the occupancy of the receptor

mediating contraction over a range of occupancies of the $5HT_2$ receptor or the α-AR. The dependencies of these parameters on the occupancy of the receptor mediating contraction could be explained by several types of functional interactions. One of the explanations for the functional interaction is based on Mackay's model in which the relaxation is affected by the stimulus for contraction (S_c) in an additive fashion. Surprisingly, S_c also affected the desensitization which led us to suggest that a kinase other than ß-ARK may be involved in the desensitization of the ß-AR.

For a given receptor-effector system, the stimulus for contraction can be varied independently of receptor occupancy by altering the efficacy of the system. The importance of studying the dependence of the ß-AR responses (i.e., relaxation and desensitization) on the efficacy of the contractile agent is that along the transduction pathway that translates receptor activation into an integrated response. Therefore, it might be possible to identify the part of the S_c (i.e. early or late in the stimulus chain) that would alter the ISO response.

The use of a series of $5HT_2$ agonists with varying efficacies: According to the model of Black and Leff (1983; see Introduction), the response elicited by different partial agonists depends on the affinity of the D•R complex for the effector. Because this property affects this response in the

same way as receptor occupancy, varying the efficacy of the contractile agonist by this method would be expected to result in alterations in ß-adrenergic response similar to those seen with varying occupancy of the $5HT_2$ receptor. In this study, the $5HT_2$ agonists were ranked by their intrinsic activities with respect to 5HT, which also corresponds to the same ranking based on the efficacy term, τ, as defined in Black and Leff (1983).

Consistent with the inverse relationship that was observed between the concentration of the contractile agonist and fractional relaxation, we find that the fractional relaxation in the presence of an agonist with lower efficacy is greater than that in the presence of a full agonist (e.g. 5HT). The finding that the fractional relaxation in rings contracted with QUIP was less than in rings contracted with 5HT was unexpected. The fractional relaxation in the presence of QUIP (**R/C**~0.6) cannot be accounted for solely on the basis of a change in efficacy. Perhaps this reflects a nonspecific effect of QUIP that may be affecting the fractional relaxation not through a change in efficacy alone.

From the data accumulated in this work, it appears that the rate constant for relaxation, $\mathbf{k_{rel}}$, does not depend on the efficacy of the $5HT_2$ agonist. This is surprising in view of the finding that $\mathbf{k_{rel}}$ decreased by increasing the occupancy of the

α-AR or $5HT_2$ receptor. The k_{rel} in the presence of BUF is indeed significantly higher than in the presence of 5HT as would be expected for an agonist of lower efficacy than 5HT. However, if we eliminate QUIP from our consideration (as it appears to have other actions as described above), we can attribute the behavior of DMT to its very low efficacy. It is expected that DMT would have a very small rate constant for the onset of the contraction response (Cory et al. 1986). If the contraction did not reach a steady state when the ISO was added, then the relaxation process could have been complicated by the simultaneous contraction. Thus, k_{rel} would be underestimated and have a large error bar associated with its value.

In summary, the changes in the relaxation response parameters that were associated with 5HT agonists of varying efficacies are consistent with changes that were observed with varying occupancy of the receptors eliciting contraction. Thus the functional interactions depend on the S_C and not only on the occupancy of the receptor mediating contraction.

The effect of varying efficacy of the contractile agonist on the desensitization response parameters. The dependence of k_{des} and t_d on the efficacy of the partial agonists eliciting contraction is as expected based on our earlier work which showed a parallel dependence of k_{des} and t_d on the

concentration of the contractile agonist (Keitz et al., paper II). Thus, an increase in contractile efficacy leads to an increase in k_{des} and a decrease in t_d. As with the ISO induced relaxation, the dependence of k_{des} and t_d on contractile efficacy demonstrate that the onset of ß-AR desensitization depends on the stimulus for activation of the contractile receptor and not merely on its occupancy.

These findings support the hypothesis that the ISO induced desensitization response in the precontracted rabbit aorta preparation might be mediated by protein kinase C (PKC). PKC requires Ca^{2+} for its activation and diacylglycerol (DG) can increase the affinity of this enzyme for Ca^{2+} (Nishizuka, 1986). In this study both the levels of Ca^{2+} and of DG were modulated by using a variety of partial agonists at the $5HT_2$ receptor. Thus, supported by the results from previous reports (Keitz et al., papers I and II), these findings suggest that PKC plays an important role in the regulation of ß-AR function.

The effect of calcium channel active drugs on the contractile response and on the ISO relaxation response parameters. Unlike the series of partial $5HT_2$ agonists which vary in the efficacy of activating the effector pathway, the calcium channel active drugs modulate a later step in the process of contraction by their effects on Ca^{2+} influx. Bay K and NIF alter Ca^{2+} influx by binding to the calcium channel and

altering the mean channel opening time; Bay K increases the mean opening time and NIF shortens the mean opening time. Consequently, Bay K enhances contraction while NIF inhibits it.

The alteration in contractile efficacy produced by Bay K or NIF is reflected in the contractile tension. Bay K significantly increased the contractile response and NIF significantly decreased it compared to control. These changes are consistent with enhancement of contractile efficacy by Bay K and attenuation of contractile efficacy by NIF.

Despite the significant changes in level of contraction that were induced by the calcium channel active agents, the studies concerning the effects of these drugs on the ß-adrenergic response remain inconclusive. Further experimental work is necessary to answer the question of whether a change in contractile efficacy by manipulating calcium entry would produce alterations in the ß-adrenergic response parameters and whether they are consistent with the changes observed with alterations in occupancy of the contractile receptors or with the use of partial 5HT agonists.

For all experiments using the calcium channel active drugs, the k_{rel} showed changes consistent with the alterations seen when efficacy was manipulated using partial agonists. In the rings contracted with PE or 5HT, the k_{rel} increased when efficacy was decreased by NIF and decreased when the efficacy

was increased by Bay K. Furthermore, the reduction in efficacy produced by NIF leads to a significant increase in **R/C** in those rings precontracted with PE. The k_{des} and t_d were also significantly altered by NIF in aortic rings precontracted with PE (table I). These results indicate that the interaction between the stimulus for contraction and relaxation interact after the entry of Ca^{2+} into the cell. Also, these findings are consistent with our conclusions from the partial agonist studies that PKC activated by Ca^{2+} may mediate desensitization by phosphorylating the ß-AR.

Figures for paper III

Figure 1:

ISO induced relaxation and desensitization in aortic rings precontracted with a series of 5HT agonists. Computer collected data points from sequential trials in a representative aortic ring showing the changes in tissue tone following the addition of 0.3 μM ISO to rings precontracted with saturating concentrations of 5HT (10 μM), QUIP (20μM), BUF (20μM) or DMT (20μM). All rings were treated with 15μM BHC to prevent agonist action on the a-adrenergic receptor. At the end of each trial all drugs were washed from the tissue by draining and replacing the buffer. There was a period of one hour between trials.

Figure 1

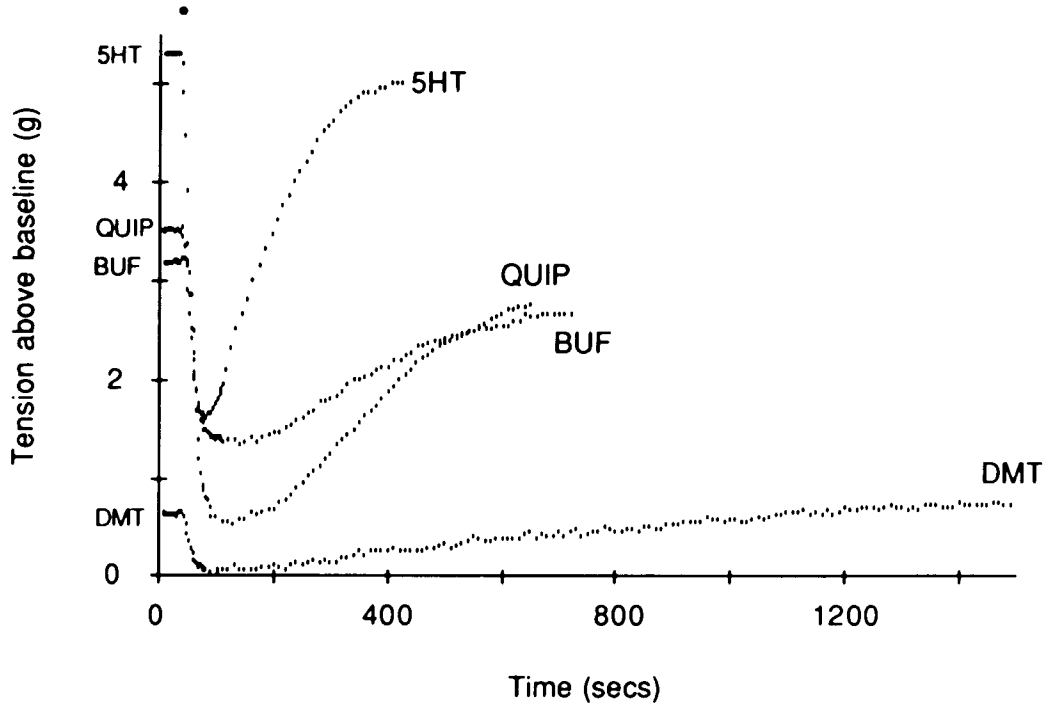

5HT
4
QUIP
BUF
2
DMT
0
Tension above baseline (g)
5HT
QUIP
BUF
DMT
0
400
800
1200
Time (secs)

Figure 2:

A: Mean fractional relaxation vs intrinsic activity of the $5HT_2$ agonist. Values presented are mean responses ± SEM from (n) repetitions from 3 rabbits for DMT and 6 rabbits for the other 3 agonists.

B: Mean k_{rel} vs intrinsic activity of the $5HT_2$ agonist. Values presented are mean responses ± SEM from (n) repetitions from 3 rabbits for DMT and 6 rabbits for the other 3 agonists.

Figure 2A

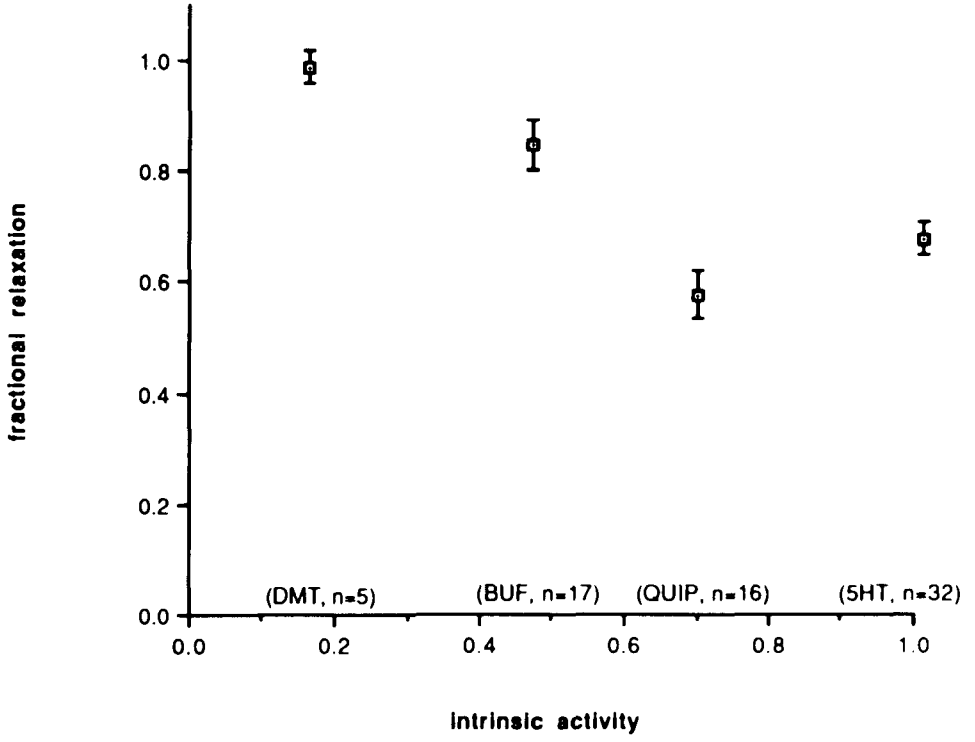


Figure 2B

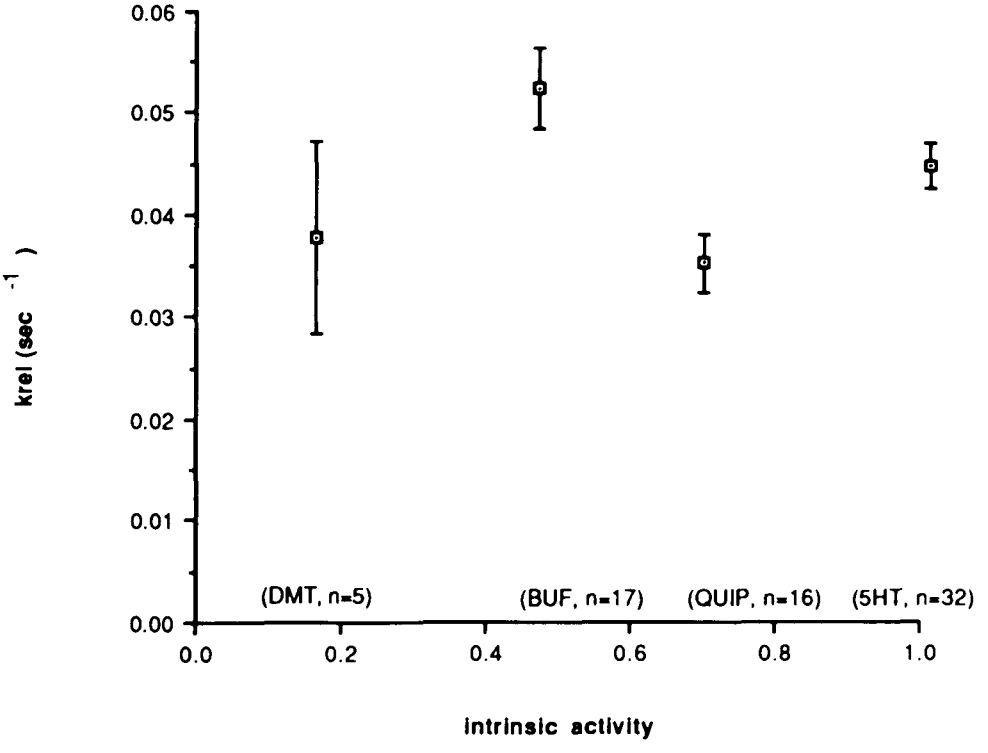


Figure 3

A: Mean fractional desensitization vs intrinsic activity of the $5HT_2$ agonist. Values presented are mean responses ± SEM from (n) repetitions from 3 rabbits for DMT and 6 rabbits for the other 3 agonists.

B: Mean k_{des} vs intrinsic activity of the $5HT_2$ agonist. Values presented are mean responses ± SEM from (n) repetitions from 3 rabbits for DMT and 6 rabbits for the other 3 agonists.

C: Mean t_d vs intrinsic activity of the $5HT_2$ agonist. Values presented are mean responses ± SEM from (n) repetitions from 3 rabbits for DMT and 6 rabbits for the other 3 agonists.

Figure 3A

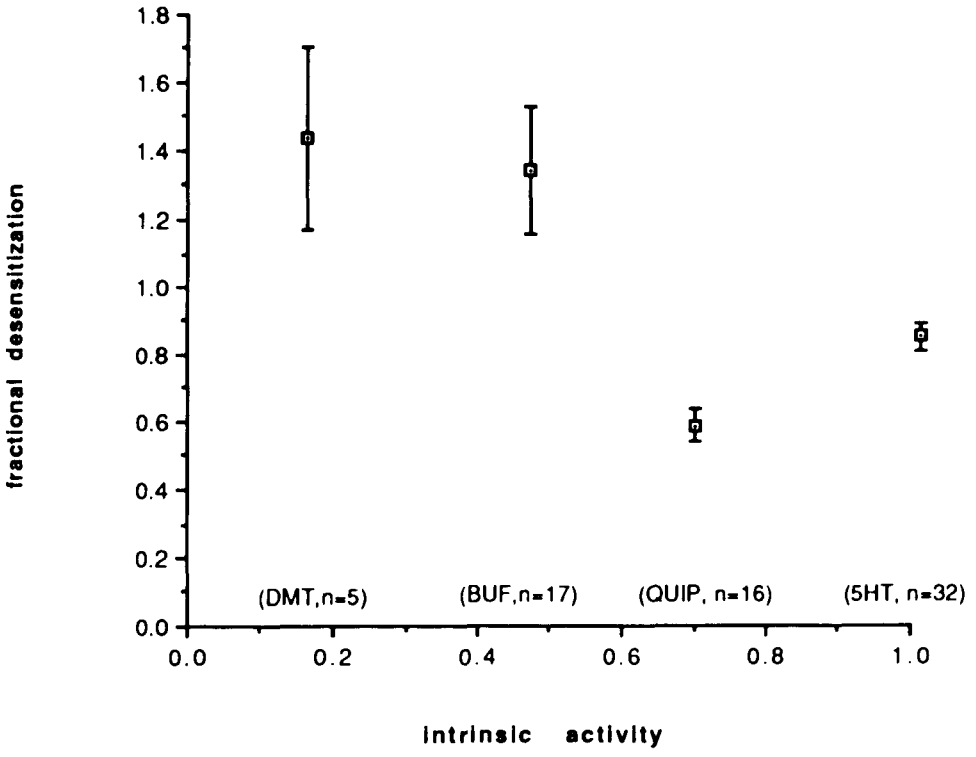


Figure 3B

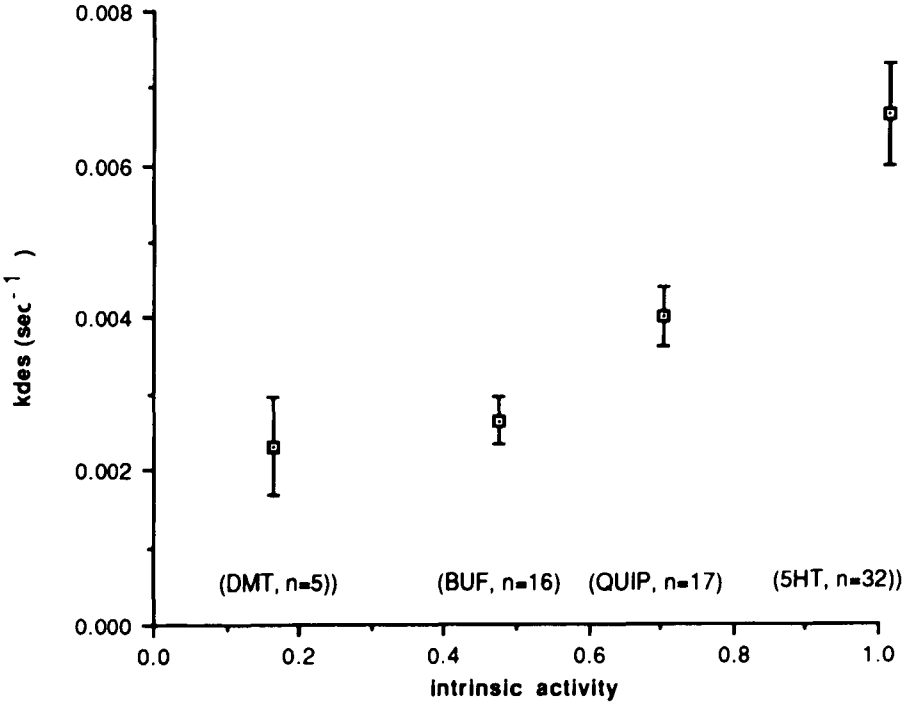


Figure 3C

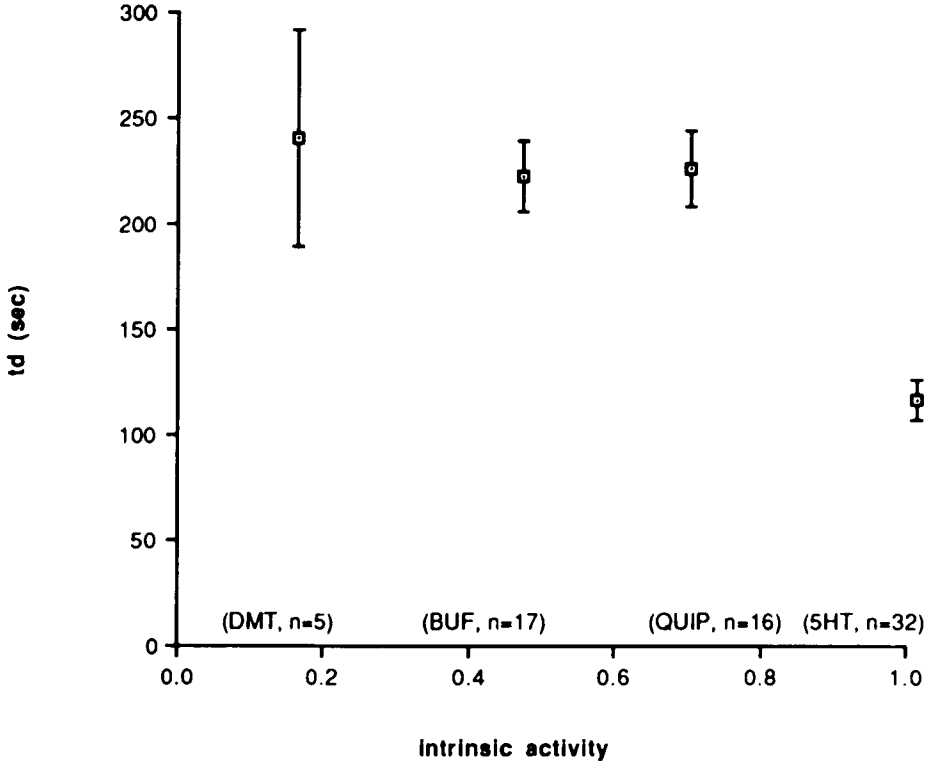

300
250
200
150
100
50
0
td (sec)
(DMT, n=5)
(BUF, n=17)
(QUIP, n=16)
(5HT, n=32)
0.0
0.2
0.4
0.6
0.8
1.0
Intrinsic activity

Table I:

Effect of NIF on PE (1 μM) and 5HT (1 μM) induced contraction and the parameters of the ß-adrenergic response. Responses to 0.3μM ISO were tested in tissue precontracted with either PE or 5HT. All rings were tested initially in the absence of NIF and subsequently following 30 minute incubation with 1 μM NIF. Parameters were estimated by fitting the computer collected data points from the observed ISO response curves to equations 1 and 2 or 3 (Methods). For all fits $r^2 > 0.97$.

*significantly different from control with $p<0.05$. All parameters were compared using a paired t-test.

Table I

	PE induced contraction		5HT induced contraction	
Parameter	**Control**	**NIF**	**Control**	**NIF**
C (g)	5.2±0.3 (n=11)	2.52±0.3* (n=11)	6.8±0.6 (n=11)	4.4±0.5* (n=11)
R/C	0.68±0.04 (n=11)	0.76±0.02* (n=11)	0.50±0.04 (n=9)	0.61±0.06 (n=9)
k_{rel} (sec^{-1})	0.037±0.003 (n=11)	0.046±0.003* (n=11)	0.048±0.002 (n=11)	0.055±0.003* (n=11)
D/R	0.80±0.27 (n=10)	0.46±0.31* (n=10)	0.80±0.4 (n=10)	0.62±0.05* (n=10)
k_{des} (sec^{-1})	$(5.0\pm0.4)\times10^{-3}$ (n=10)	$(4.0\pm0.3)\times10^{-3}$* (n=10)	$(8.0\pm1.0)\times10^{-3}$ (n=10)	$9.0\pm1.0)\times10^{-3}$ (n=10)
t_d (sec)	225±13 (n=10)	263±18* (n=10)	71±13 (n=10)	62±16 (n=10)

Figure 4:

Effect of NIF on the response to ISO in a ring precontracted with 1 μM PE. Points shown are computer collected data points (see Methods) and were fitted to equations 1 (for the relaxation responses) and 2 (for the desensitization response). In the examples shown, $r^2 \geq 0.99$ for all fits. The average values of the fitted parameters, R, k_{rel}, D, k_{des} and t_d under these conditions are summarized in the table I.

Figure 4

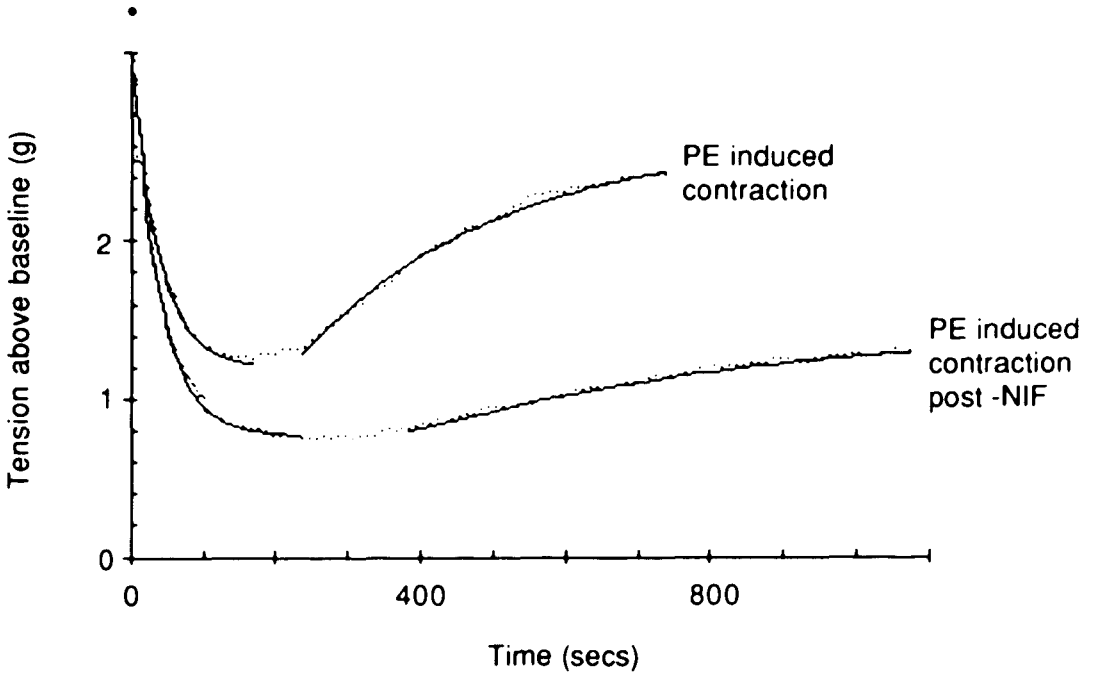

Tension above baseline (g)
2
1
0
0
400
800
Time (secs)
PE induced contraction
PE induced contraction post -NIF

Table II:

Effect of Bay K on PE (0.1 μM) and 5HT (0.06 μM) induced contraction and the parameters of the ß-adrenergic response. . All rings were tested initially in the absence of Bay K and subsequently following 30 minute incubation with 0.3 μM Bay K. Parameters were estimated by fitting the computer collected data points from the observed ISO response curves to equations 1 and 2 or 3 (Methods). For all fits $r2 > 0.97$.

*significantly different from control with $p<0.05$. All parameters were compared using a paired t-test.

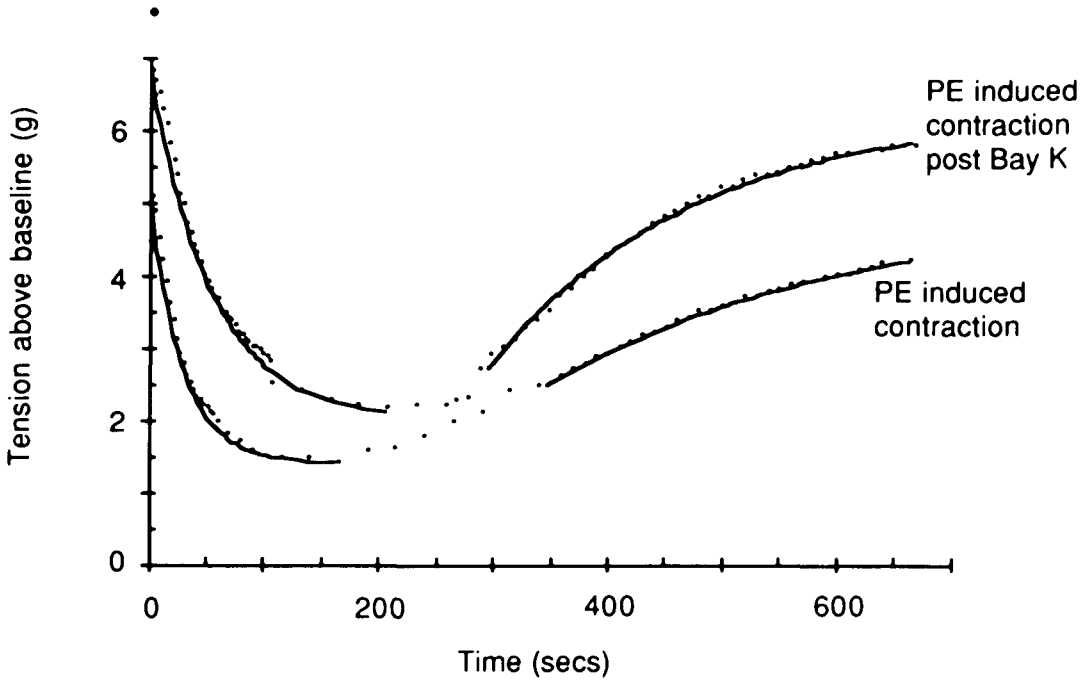
Table II

	PE induced contraction		5HT induced contraction	
Parameter	Control	Bay K	Control	Bay K
$C(g)$	4.5±.4 (n=16)	6.1±.6* (n=16)	4.3±0.7 (n=5)	6.0±0.9* (n=5)
R/C	0.69±.04 (n=14)	0.72±.04 (n=14)	0.75±0.07 (n=5)	0.63±0.04 (n=5)
k_{rel} (sec^{-1})	0.04±0.002 (n=13)	0.024±0.002* (n=13)	0.04±0.01 (n=5)	0.031±0.007* (n=5)
D/R	1.07±0.04 (n=7)	0.87±0.04* (n=7)	1.12±0.06 (n=5)	1.22±0.08 (n=5)
k_{des} (sec^{-1})	$(3.0\pm0.3)\times10^{-3}$ (n=7)	$(7.0\pm2.0)\times10^{-3}$ (n=7)	$(4.0\pm1.0)\ *10^{-3}$ (n=5)	$(5.0\pm1.0)*10^{-3}$ (n=5)
t_d (sec)	174±29 (n=8)	274±40 (n=8)	139±36 (n= 5)	177±16 (n=5)

Figure 5:

Effect of Bay K on the response to ISO in a ring precontracted with 0.1 μM PE. Points shown are computer collected data points (see Methods) and were fitted to equations 1 (for the relaxation responses) and 2 (for the desensitization response). In the examples shown, $r^2 \geq 0.99$ for all fits. The average values of the fitted parameters, R, k_{rel}, D, kdes and td under these conditions are summarized in the table II.

Figure 5


Tension above baseline (g)
6
4
2
0
0
200
400
600
Time (secs)
PE induced contraction post Bay K
PE induced contraction

DISCUSSION.

Summary of results and conclusions:

1. The response to ISO can be accurately described by 5 parameters: **R**, the magnitude of relaxation, **D**, the magnitude of desensitization, the observed rate constants of these two processes, k_{rel} and k_{des}, and an additional kinetic parameter that describes the delay in the onset of desensitization, t_d.

2. In order to compare different rings and because the relaxation depends on the contraction and cannot exceed it, the extent of relaxation is described as fractional relaxation, **R/C**. Likewise, the desensitization is represented as fractional desensitization, **D/R**.

3. Only the relaxation response parameters were dependent on the concentration of ISO at a fixed concentration of either PE or 5HT. Both fractional relaxation and k_{rel} showed a saturable dependence on the concentration of ISO, whereas the three desensitization parameters were independent of ISO concentration.

4. The response to a constant concentration of ISO was quantitatively different in the presence of equiactive concentrations of PE, 5HT, HA or KCl.

5. All parameters were altered by changes in concentration of the agonists eliciting contraction. At a fixed concentration of ISO, **R/C** showed a steep concentration

dependence on the concentration of PE. **R/C** decreased from ~1.0 to ~0.2 as PE concentration increased. Alternatively there was minimal change in **R/C** (~0.7) over a similar range in 5HT concentrations. The rate constant for relaxation was decreased with increasing concentration of either PE or 5HT.

The fractional desensitization showed little change over a range of PE or 5HT concentrations, whereas the kinetic parameters k_{des} and t_d showed a saturable dependence on the concentration of contractile agonist. The k_{des} increased with increasing concentration of PE or 5HT whereas the t_d decreased with increasing concentration of contractile agonist.

6. The relaxation response was altered by changing the efficacy of the contractile agonist. The fractional relaxation was greater in tissues precontracted with the $5HT_2$ partial agonists (DMT and BUF) than in tissues precontracted with 5HT. **R/C** was also greater in rings contracted with PE following NIF treatment than in control rings contracted with PE alone. The k_{rel} was decreased by increasing efficacy with the calcium channel activator, Bay K in the presence of either PE or 5HT and increased when NIF was used to decrease the contractile efficacy.

7. Both the rate constant for desensitization and the t_d were altered by a change in contractile efficacy using various

$5HT_2$ agonists. The k_{des} was greatest for 5HT and less for DMT and BUF whereas the t_d was smallest for 5HT and greatest for DMT and BUF.

8. The saturable dependence of k_{rel} on the concentration of ISO suggested that the rate-limiting step of the relaxation process was activation of an effector by the drug-receptor complex.

9. We conclude that the regaining of tension that follows ISO induced relaxation is an attenuation of response due to ß-AR desensitization.

10. The finding that the fractional desensitization was minimally altered by all the conditions tested in this work while the rate constant for desensitization and t_d were altered by changing conditions demonstrates that the kinetic parameters are more suitable for characterizing a desensitization process in this system.

11. We conclude that the sole determinant of the response to ISO in precontracted tissue is not absolute level of tissue tone as the responses were different in rings precontracted to the same level of tone by four different protocols.

12. We conclude from the lack of dependence of the kinetic desensitization parameters, k_{des} and t_d, on the concentration of ISO and the dependence of these parameters

on the concentration of PE and 5HT that the desensitization in this system is unlikely to be primarily mediated by ß-ARK. We suggest that the desensitization is mediated by a stimulus produced by the contractile agonist, perhaps by phosphorylation by PKC.

Discussion:

The dependence of the ß-adrenergic response parameters on contractile efficacy is an illustration of functional interactions in a smooth muscle system: the *stimulus for contraction* (S_C) elicited by activation of the α-AR or $5HT_2$ receptor affects the *relaxation* as well as the *desensitization* induced by the action of ISO at the ß-AR. This work provides an interesting perspective about simultaneous activation of distinct membrane receptors in a functional system because it illustrates different modes of interactions between receptors and effectors. Often, functional interactions are thought to occur between stimuli produced by different effector systems. One classical example of this type of functional interaction is illustrated in this paper and in papers I and II as the functional antagonism between the ISO induced relaxation of an aortic ring and the contraction elicited by PE via the α-AR. This functional antagonism is thought to occur via interactions at the level of the stimulus chain; i.e. the interactions occur *after* receptor activation or between the respective stimuli (Keitz et al., paper II). In contrast, the modulation of ß-AR desensitization is likely to occur via phosphorylation of the ß-AR by PKC (paper II). Activation of PKC is elicited by the stimulus for contraction acting at two different stages of the response generation process; at an early stage through the

activation of the receptor and generation of DG and at a later stage through the modulation of Ca^{2+} levels in the cell. This represents an attenuation of receptor function by the action of a stimulus on this receptor.

There is evidence in the literature to support the notion of a stimulus altering the properties of functionally antagonistic receptors. For example, the purified catalytic subunit of PKC is able to phosphorylate the purified $ß_2$-AR and the purified catalytic subunit of PKA can phosphorylate the α_1-AR (Bouvier et al., 1987). Furthermore, the activation of muscarinic M_1 receptors in dissociated cardiac myocytes, which are coupled to PI metabolism, induces time and concentration dependent redistribution of $ß_1$-ARs from the membrane to the cytosol fraction (Constantinos and Limas, 1985). This finding is consistent with internalization of the ß-ARs. In the present study PKC is activated as part of a contractile response in an *intact tissue* and all parameters of the ß-adrenergic response are described as functional responses reflected as changes in tissue tone. This demonstrates the physiologic relevance of such stimulus:receptor crosstalk.

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