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THE LINK BETWEEN AGONIST ACTION AND RESPONSE IN SMOOTH MUSCLE[1]

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A powerful excitatory agent placed in the immediate environment of a smooth muscle will induce or accelerate a series of cellular reactions. Usually, the first step in the chain of reactions is the formation of a reversible complex between molecules of the agonist and specific receptors in the muscle's plasma membrane. The end result is a change in the tension or the length of the smooth muscle fibers, or both. The central focus in this review will be on the sequence of cellular reactions that appear to be essential for linking the interaction of the drug and receptor to the activation of the contractile apparatus.

THE DRUG-RECEPTOR INTERACTION AND ITS RELATION TO THE RESPONSE

During the past several years a number of excellent reviews dealing with the nature of the pharmacological receptor and the consequences of its interaction with excitatory and inhibitory agents have appeared in the literature (1–10). Only those aspects of the subject that relate to the central theme of this review will be considered here.

The concept that a drug induces an effect or elicits a response by interacting with highly specific receptor areas in the target cell has been expressed by such notable investigators as Ehrlich (11, 12), Langley (13), and Clark (14, 15). Furchgott (4) has defined the receptor area as a specific molecular site or structure in (or on) an effector cell with which molecules of a specific agonist (excitatory agent) must react in order to elicit the characteristic response of the cell to the agonist. According to Schueler (16) a receptor is a pattern of forces forming a part of some biological system and which is complementary to some pattern of forces presented by the drug or hormone molecule, such that an interaction between the two patterns may occur. These definitions attest to our uncertainties concerning the exact nature of the receptor.

In spite of the difficulties encountered in studies designed to uncover fundamental characteristics of receptors, a number of workers have at-

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tempted to shed some light on the subject by venturing forth with hypothetical models of pharmacological receptors. Waser (17) visualizes the cholinergic receptor site as a membrane pore with anionic sites on its periphery. These anionic sites control the flow of ions across the membrane. Quaternary cations, by interacting with the anionic sites, widen the pore opening. The increased flow of ions that results leads to a chain of events which culminate in a functional response. Antagonists, such as *d*-tubocurarine, may hinder the passage of ions by blocking access to the pore from the extracellular region (17, 18).

Watkins (19) conceived of the cholinergic receptor as a lipo-protein complex. The lipid portion is alleged to be a choline-containing molecule which may be lecithin or sphingomyelin. When the receptor is exposed to an excitatory drug such as acetylcholine, the drug will compete with the lipid for its site of attachment to the protein, and may displace it from the protein. Displacement of the lipid will induce a conformational change in the protein and thereby increase the permeability of the membrane to various ions.

Csillik (20) believes that the cholinergic receptor consists of expanded polypeptide chains with lipid particles transversely attached to them (lipoprotein). Under resting conditions, the structure is permeable only to small ions such as K^+, which regulate the membrane potential. When the receptor interacts with acetylcholine, the protein structure becomes distorted. As a consequence, some of the attached lipids change from a transverse to a longitudinal orientation. The resulting irregularity in the molecular framework of the membrane gives rise to openings that are wide enough to permit the passage of larger ions. The increased ionic flux leads eventually to a response.

Belleau (6) has postulated that the cholinergic receptor behaves as an enzyme. Although he has never specified the reaction that it catalyzes, he maintains that the enzymatic activity of the receptor is modified by a conformational change which results from the interaction between the receptor and the drug. The increased activity gives rise to a stimulus and the stimulus induces a response.

The cholinergic receptor is not the only one to enjoy the distinction of being labelled or being associated with an enzyme. Robison et al (5, 21) have advanced the concept that the β adrenergic receptor and, in some tissues, the α adrenergic receptor are closely linked to the enzyme adenyl cyclase. This enzyme catalyzes the breakdown of ATP to adenosine 3′,5′-monophosphate (cyclic AMP). It is presumed that the α and β adrenergic receptors may be separate regulatory sub-units linked to the enzyme molecule. When catecholamines and other drugs react with the α or β adrenergic receptors they produce conformational changes that modify the activity of the adenyl cyclase. This results in an increase or a decrease in the level of cyclic AMP in the cell. The cyclic AMP has been termed a second messenger. One of its functions is thought to be the regulation of cellular

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reactions that lead to a contraction or relaxation of muscle fibers. Precisely what reactions it regulates in this regard is still not clear. Nonetheless, an increased level of cyclic AMP appears to correlate with an increased inotropic effect in cardiac muscle (5, 22–24) and with an inhibition of contraction in smooth muscle (25, 26).

In a somewhat different vein Belleau (27, 28) and Bloom & Goldman (29) maintain that the β adrenergic receptor is synonymous with an adenylcyclase-ATP complex. The catecholamines are assumed to bind directly with the active center of the complex. In so doing, the hormones initiate the hydrolysis of ATP. This in turn initiates the series of reactions that induce a response.

Belleau (28) and Bloom & Goldman (29) have also elaborated on the α adrenergic receptor. They hypothesize that this receptor is an enzyme-substrate complex, consisting of ATP and an ATPase. When the neurohormone complexes with the enzyme, ATP is hydrolyzed. This reaction mediates the release of bound calcium, and the released calcium serves to activate the contractile elements. Belleau (27) and Bloom & Goldman (29) differ with regard to the location of the α receptor. Belleau is of the opinion that the enzyme may be a Na-K activated ATPase that is located in the membrane and is involved in ion transport. Bloom & Goldman believe that the enzyme hydrolyzes meromyosin-bound ATP and that its site of action is intracellular.

The work briefly touched upon above serves to illustrate the proposals that have been made with respect to the functional nature of the receptor. Although these proposals are interesting and attractive, the speculative aspect of this area of research is still considerable.

A. J. Clark (14, 15, 30, 31) was one of the first to relate the association between the drug and receptor to the ultimate response of the cell in quantitative terms. He pictured a drug reacting with a specific receptor in a rapid, reversible manner in accordance with mass law. Moreover, he suggested, although with some reservations, that the magnitude of the response was proportional to the number of receptors occupied by the drug. His postulates may be expressed as follows:

$$D + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR \xrightarrow{k_3} \text{Response} \qquad 1.$$

where D is the concentration of free drug in the extracellular environment of the cell, R is the concentration of free (unbound) receptors in the cell, DR is the concentration of drug-receptor complex formed, Response is the magnitude of the muscle contraction or some other functional change that occurs, k_1 and k_2 are reaction rate constants, and k_3 is a proportionality constant that equates the magnitude of response with the concentration of drug-receptor complex.

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Under equilibrium conditions the mass law expression for the dissociation of the drug-receptor complex is:

$$\frac{(D)(R)}{(DR)} = \frac{k_2}{k_1} = K_{DR} \qquad 2.$$

where K_{DR} is the dissociation constant.

In many instances the amount of drug bound to the receptors appears to be a negligible quantity compared with the total amount of drug introduced into the environment of the cell. That is to say, the concentration of free (unbound) drug is essentially equal to the concentration of total (bound+ unbound) drug present. Conversely, the fraction of total receptors bound to drug may not be neglected; and R_T, the total concentration of receptors in the cell is always taken to be the sum of R, the concentration of free receptors and DR, the concentration of bound receptors. The mass law expression may then be written:

$$\frac{(D)(R_T - DR)}{(DR)} = K_{DR} \qquad 3.$$

where D refers to the concentration of free or both free and bound drug present. Solving for (DR) gives:

$$DR = \frac{(R_T)(D)}{K_{DR} + (D)} \qquad 4.$$

Since the magnitude of the response is assumed to be equal to $k_3(DR)$, the equation becomes:

$$\text{Response} = \frac{k_3(R_T)(D)}{K_{DR} + (D)} \qquad 5.$$

This equation, the derivation of which is based on Clark's postulates and approximations, defines the quantitative relationship that one should expect to find between the magnitude of the response and the concentration of drug introduced. It indicates that the response will vary as a rectangular hyperbolic function of the drug concentration. Clark found many examples in the laboratory that proved to be consistent with his postulated model (31).

According to Clark's hypothesis, differences in potencies among various agonists (excitatory agents) are determined exclusively by their respective affinities for the tissue receptors. A drug that has a strong affinity for the receptors will saturate them at a relatively low concentration and is therefore considered to be a potent agent. The reverse will be true for a drug that has a weak affinity for the receptors.

Subsequently, the concept of a second determinant of potency was introduced. Ariens, who first called attention to this second factor, labelled it intrinsic activity (32-34). It may be defined as the relative intensity with which

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a drug evokes a response once the drug has combined with the receptors. In essence Arien's model is much like Clark's model except that the value of the proportionality constant (k_3) relating the magnitude of response to the concentration of drug-receptor complex is postulated to be different for different drugs. This would mean that agonists with high intrinsic activities have the capacity to elicit larger maximal responses than do agonists with low intrinsic activities. A drug that possesses a high affinity for the receptors and lacks intrinsic activity serves as an effective blocking agent.

In the mid-fifties the investigations of Furchgott (35, 36), Stephenson (9), and Nickerson (37, 38) led to a radical departure from previous interpretations of dose-response relationships. These workers accepted Clark's model up to the point where the drug-receptor complex was formed. They rejected the idea that the response was proportional to the number of receptors occupied by the drug. However, they did not abandon the thesis that each receptor occupied by an agonist evokes a quantum of excitation or change in the cell. Stephenson, without speculating about the type of cellular change or excitation that was induced, simply called it stimulus (9). It was assumed that the magnitude of the stimulus induced would be proportional to the number of receptors filled with an agonist. A proportionality constant analogous to Ariën's intrinsic activity (32) was also incorporated. Furchgott (39) used the term intrinsic efficacy to denote the relative intensity with which a drug, by interacting with its receptors, would evoke the stimulus. Although the ultimate functional response of the effector cell is presumed to be generated by the stimulus, it does not necessarily have to be a linear function of the stimulus. These concepts may be expressed as follows:

$$D + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR \xrightarrow{\epsilon} S \xrightarrow[\text{function}]{\text{complex}} \text{Response} \qquad 6.$$

and

$$\text{Response} = f(S) \qquad \left(f \frac{\epsilon[R_T][D]}{K_{DR} + [D]}\right) \qquad 7.$$

where f refers to some complex function, ϵ is the intrinsic efficacy, and S is the magnitude of the stimulus. Stephenson (9) expressed these same concepts in a slightly different manner. He used a proportionality constant which he called efficacy. This term proved to be equal to the product of ϵ and R_T.

There were two major reasons for replacing Clark's model for this newly conceived, more complex model. First, Stephenson as well as other investigators found that the configuration of dose-response curves did not always define a rectangular hyperbolic relationship between the magnitude of the response and the concentration of the agonist. The diverse shapes of the experimental curves seemed to be determined by a complex series of

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reactions and factors that defied simple interpretation. Second, in experiments performed with poorly reversible blocking agents, the β-haloalkylamines, it was shown that the maximum amplitude of contraction produced by some agonists in smooth muscle fibers was not modified when some portion of the receptor population was blocked (39). This suggested that certain powerful excitatory agents have such high intrinsic efficacies, they can induce a muscle to perform to its maximum capacity without having to occupy all the available receptors (9). It has been estimated that, in some instances, a maximum response can be elicited when only 1% of the available receptors are occupied (3, 37).

The powerful excitatory agents that elicit large maximum responses have been labelled full agonists (9). Those drugs that induce a small stimulus and response even after saturating all the receptors with which they interact are presumed to have low intrinsic efficacies and are called partial agonists (9). As one might expect, compounds that have an appreciable affinity for a group of receptors and are devoid of any intrinsic efficacy act as blocking agents (antagonists) (9).

The new developments that were uncovered by Stephenson, Furchgott and other investigators in the area of drug-receptor interactions have tended to dissuade attempts to attach a significance to the shapes of dose-response curves. As the model described above indicates, there is general agreement that the concentration of the drug-receptor complex formed varies as a rectangular hyperbolic function of the concentration of the agonist introduced. This relationship is assumed to reflect the operation of the mass law governing the reversible association between the agonist and its receptors. The link between the response and the level of agonist is another matter. Based on evidence presently available, it is reasonable to assume that a multiplicity of factors determine the relationship between the response of a tissue and the concentration of agonist to which the tissue is exposed. The physico-chemical characteristics of the drug-receptor interaction represent only one of these factors. As a consequence a precise interpretation of the shape of a dose-response curve is difficult to justify.

In spite of the validity of these arguments, the observation made by Goldstein et al cannot be overlooked (40). These authors point out that an unusually large number of physiological (and biochemical) responses in a diverse population of biological systems have been found to vary as a rectangular hyperbolic function of the concentration of the activating agents that initiate them. These include contractions of the cat spleen (41), the rabbit aorta (42), the guinea-pig ileum (43), and the frog rectus abdominis (44) as well as blood pressure changes in the dog (45) and voltage changes in the neuromuscular junction of the frog sartorius muscle (46). In view of the infinite variations that are possible, a preponderance of one type of dose-response relationship would suggest that some specific rate-limiting process is operative in all these biological systems. Goldstein et al (40) have suggested that the hyperbolic dose-response curve represents a

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saturation phenomenon governed by the mass law. If so, it would preclude the possibility of a drug-receptor interaction having a strong influence on the shape of the curve. How could an agonist that produces a maximum response by occupying only a small portion of the available receptors yield data representing a saturation phenomenon? Still, a number of powerful agonists have been found to exhibit a hyperbolic relationship to the responses they elicit. Needless to say it would be of great interest to find some way of reconciling and integrating the individual observations and interpretations that have been made by the various investigators that have contributed to this area of research.

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One very attractive approach to the problem has been introduced by Furchgott (39). He demonstrated, in purely mechanistic terms, that Clark's postulated scheme is not the only one that will yield a rectangular hyperbolic dose-response curve. To put it more specifically, the magnitude of the response need not be directly proportional to the number of receptors filled with drug in order to establish a hyperbolic relationship between response and drug concentration. Such a relationship would also be observed if the response, instead of being a linear function, were a hyperbolic function of the number of drug-filled receptors. The latter fact gives one considerably wider latitude in constructing a physico-chemical model that is consistent with the observed experimental data. Furchgott (39) showed that the relationship in question would hold if the functional response of a tissue were elicited at the end of a long chain of cellular reactions each of which obeyed the mass law. This concept may be expressed in the following manner:

$$D + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} DR$$

$$DR + P_1 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} P'_1 \qquad 8.$$

$$P'_1 + P_2 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} P'_2$$

etc. until

$$P'_{n-1} + P_n \underset{k_n}{\overset{k_x}{\rightleftharpoons}} \text{Response}$$

where P_1, P'_1, P_2, P'_2, P'_{n-1}, P_n represent reactants or products of the above reactions as indicated. It may be seen that in each individual step in the sequence above (except for the first reaction) one of the two reactants involved is the product of the preceding reaction. With such a model in operation, neither the introduction of a full agonist that occupies a small fraction of the total receptors nor the introduction of a partial agonist

would necessarily modify the strict adherence of the dose-response curve to its rectangular hyperbolic configuration.

One might reasonably inquire as to the extent to which Furchgott's mechanistic model characterizes the true situation in the living cell. This is, of course, difficult to evaluate at present. However, there are a number of cellular processes that are assumed to occur in the functioning smooth muscle fiber that might very well be part of such a series of reactions. The first of these is obviously the reversible interaction between the agonist introduced into the extracellular environment of the muscle and the tissue receptors in the fiber membrane. A second essential process which may or may not be next in line, is the inward movement of calcium into the cytoplasm of the muscle fiber (47–50). This may be assumed to occur via a calcium transport system located in the fiber membrane. A third cellular reaction would be the reversible interaction between free cytoplasmic calcium and active centers in the contractile apparatus. This interaction would then be followed by a change in the length or tension, or both, of the smooth muscle fibers.

The question is, how are these essential cellular reactions in the smooth muscle fiber coupled to each other? One plausible model that is consistent with the experimental observations that have been made may be represented as follows:

$$D + R \underset{k_0}{\overset{k}{\rightleftharpoons}} (DR) \xrightarrow{k_x} T_T \qquad 9.$$

$$Ca_o + T \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (Ca_oT) \xrightarrow{k_3} Ca_i \xrightarrow{k_{ex}} [\text{sink}] \qquad 10.$$

$$Ca_i + M \underset{k_5}{\overset{k_4}{\rightleftharpoons}} (Ca_iM) \xrightarrow{k_6} [\text{Response}] \qquad 11.$$

Where: D is the concentration of agonist.

R is the concentration of unoccupied tissue receptors.

(DR) is the concentration of the drug-receptor complex.

T_T is the total concentration of active calcium transport sites in the membrane.

Ca_o is the extracellular concentration of calcium ions.

T is the concentration of free (unbound) calcium transport sites.

(Ca_oT) is the concentration of the complex formed by the reaction between calcium ions and active calcium transport sites.

Ca_i is the concentration of free cytoplasmic calcium ions.

M is the concentration of free (unoccupied) active centers in the contractile apparatus.

(Ca_iM) is the concentration of the complex formed by the reaction between cytoplasmic calcium and active centers in the contractile apparatus.

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[sink] is the depot where the calcium ions that are actively extruded from the cytoplasm are deposited.

[Response] is the magnitude of the contraction that the muscle develops.

k, k_o, k_1, k_2, k_3, k_{ex}, k_4, and k_5 are reaction rate constants.

k_x and k_6 are proportionality constants.

The initial reaction in the three step model represented above is the reversible interaction between the agonist and the tissue receptors. It is assumed that the drug-receptor complex formed by this interaction leads directly or indirectly to the activation of a calcium transport system. The molecular mechanism by which this is accomplished is not known. Nor is the nature of the calcium transport system clearly defined. However, it appears to be a saturable system. It may consist of a finite number of sites on which calcium must be reversibly adsorbed before entering membrane pores, or a finite number of carrier molecules which ferry the calcium inward. The number of adsorption sites or carrier molecules that become operative is presumably related to the number of receptors that complex with the agonist. In the scheme above the concentration of active calcium transport sites is represented as being proportional to the concentration of drug-receptor complexes formed, although some other relationship may be equally plausible.

The next step consists of a rapid reversible association between calcium ions originating in a cellular depot or in the extracellular fluid and unoccupied sites in the calcium transport system. It is assumed that the calcium ions bound by the transport system are delivered to the membrane-cytoplasm interface where the divalent ions are then released and become part of the free calcium ion concentration in the cytoplasm. The movement of calcium across the membrane via the transport system is pictured as being exclusively in the inward direction. This representation of the transport process seems appropriate because the concentration of calcium in the cytoplasm in relation to its affinity for the transport system is probably too low for the divalent ions to complex more than an insignificant fraction of the adsorption sites or carrier molecules at the inner surface of the membrane. However, excessive accumulation of calcium in the cytoplasm is supposedly prevented by the continuous operation of a metabolically dependent calcium extrusion system (51–54).

The third step in the model is the reversible reaction between calcium ions in the cytoplasm and active centers in the contractile machinery. The calcium-protein complex formed from this reaction generates the molecular changes that lead to a shortening or an increase in tension of the muscle fiber. The magnitude of the contractile response is shown to be directly proportional to the number of calcium-protein complexes ($Ca_i M$) formed. As an alternative possibility one could incorporate a concept enunciated by Furchgott (39, 36). He visualized a dynamic equilibrium existing between the concentration of contractile proteins in the relaxed state and the con-

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centration of contractile proteins in the contracted state. The response of the muscle would, of course, be related to the concentration of proteins in the contracted state. Thus:

$$C_r \underset{k_8}{\overset{k_7}{\rightleftharpoons}} C_c \xrightarrow{k_9} \text{Response} \qquad 12.$$

where C_r is the concentration of contractile proteins in the relaxed state, C_c is the concentration of contractile proteins in the contracted state, k_7 and k_8 are reaction rate constants, and k_9 is a proportionality constant. Assuming that the concentration of the calcium-protein complex enhances the rate of the reaction to the right, the quantity (Ca_iM) may be substituted for the constant k_7. The sequence of cellular changes would then be expressed as follows:

$$C_r \underset{k_8}{\overset{(Ca_iM)}{\rightleftharpoons}} C_c \xrightarrow{k_9} \text{Response} \qquad 13.$$

In such a sequence of cellular changes, the response would be a rectangular hyperbolic function of (Ca_iM) rather than a linear function. It should be noted, however, that replacing one relationship with the other in the model would not materially affect the basic concepts that are to be discussed below.

Many smooth muscles can be stimulated to develop relatively sustained submaximal contractions. This would suggest that the series of cellular reactions associated with the formation of the drug-receptor complex, the inward movement of calcium, and the initiation of the mechanical response can approach equilibrium or a steady state, or both. Under these conditions the various quantities taking part in the reactions described above may exhibit the following interrelationships:

$$\text{Response} = K_6(Ca_iM) \qquad 14.$$

$$(Ca_iM) = \frac{M_T(Ca_i)}{K_M + (Ca_i)} \qquad 15.$$

where (M_T) equals the total concentration of active centers in the contractile machinery and K_M equals k_5/k_4.

$$Ca_i = \frac{k_3}{k_{ex}}(Ca_oT) \qquad 16.$$

$$(Ca_oT) = \frac{T_T(Ca_o)}{K_T + (Ca_o)} \qquad 17.$$

where K_T equals k_2/k_1.

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$$T_T = k_x(DR) \tag{18.}$$

$$DR = \frac{R_T \cdot D}{K_{DR} + D} \tag{19.}$$

where R_T equals the total concentration of receptor sites and K_{DR} equals k_o/k. By making the proper substitutions in the equations above and simplifying, it is possible to derive an expression that relates the magnitude of the response to the concentration of agonist in the external medium. The expression is:

$$\text{Response} = \frac{[Ca_o k_6 M_T k_3 k_x R_T]D}{k_{ex}K_M K_{DR} K_T + k_{ex}K_M K_{DR} Ca_o + [k_{ex}K_M K_T + k_{ex}K_M Ca_o + Ca_o k_3 k_x R_T]D} \tag{20.}$$

This expression discloses a rectangular hyperbolic relationship between the mechanical response of the muscle and the concentration of agonist in the external medium. By taking the reciprocals of both sides of the equation one obtains the following expression for a straight line:

$$\frac{1}{\text{Response}} = \frac{K_{DR}}{k_6 M_T}\left[\frac{K_M k_{ex}}{k_3 k_x R_T}\left(\frac{K_T}{Ca_o} + 1\right)\right]\frac{1}{D} + \frac{1}{k_6 M_T}\left[\frac{K_M k_{ex}}{k_3 k_x R_T}\left(\frac{K_T}{Ca_o} + 1\right) + 1\right] \tag{21.}$$

If $\frac{K_M k_{ex}}{k_3 k_x R_T}\left(\frac{K_T}{Ca_o} + 1\right)$ is made equal to P

then

$$\frac{1}{\text{Response}} = \left(\frac{K_{DR} P}{k_6 M_T}\right)\frac{1}{D} + \frac{1}{k_6 M_T}(P + 1) \tag{22.}$$

There are a number of interesting features of the straight line equation that are worthy of mention. One of these is the observation that an inhibitory agent that modifies the value of a constant in the P term can have a number of different inhibitory effects. This would be true of the β-haloalkylamines which, by irreversible blockade, reduce the magnitude of R_T and thereby increase the value of P. If P happened to be very much smaller than 1, a moderate increase in its value would change the slope of the line but not the intercept. The blocking agent that produced this change would appear to be a competitive inhibitor. If P were very much larger than 1, a moderate increase in its value would change both the slope and the intercept of the straight line by the same factor. The antagonist that modified P would then appear to be a noncompetitive agent. At intermediate values of P the effect of the inhibitory agent would appear to be partly competitive and partly noncompetitive. This analysis would also apply to inhibitory

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agents that altered some of the other constants in the P term. For example a drug that accelerated the extrusion of calcium from the cytoplasm would fall into this category. The only inhibitory agent that would be consistently competitive would be one that vied with the agonist for the tissue receptor or modified in some way the value of K_{DR}. The only antagonist that would be consistently noncompetitive would be one that somehow reduced the number of active centers (M_T) in the contractile machinery or reduced the value of the proportionality constant (k_6) that relates response to the number of active centers filled with calcium (Ca_iM).

The straight line equation also discloses a simple method for converting a full agonist into a partial agonist. The equation for the dose-response curve generated by a full agonist invariably has a P term of low magnitude ($P<1$). The magnitude of the P term can be increased appreciably simply by lowering the calcium ion concentration in the external medium or in the cellular depot. Lowering the calcium ion concentration will, in effect, reduce the degree of contraction that the agonist can produce by occupying a fixed number of receptors. More receptors will now need to be occupied to achieve the same end result. Theoretically, if one were to lower the calcium concentration to the point where a further reduction would increase the slope and intercept of the straight line by the same factor, he would be in a zone in which the concentration of agonist that produced a half maximal response would closely approximate the value of the dissociation constant for the drug-receptor complex.

The equation may also be rearranged so that the external concentration of calcium ions rather than the concentration of agonist is the independent variable. The expression is:

$$\frac{1}{\text{Response}} = \frac{K_T}{k_6 M_T}\left[\frac{K_M k_{ex}}{k_3 k_x R_T}\left(\frac{K_{DR}}{D}+1\right)\right]\frac{1}{Ca_o}+\frac{1}{k_6 M_T}\cdot\left[\frac{K_M k_{ex}}{k_3 k_x R_T}\left(\frac{K_{DR}}{D}+1\right)+1\right] \qquad 23$$

By replacing $\dfrac{K_{DR}+D}{k_x R_T D}$ with its equivalent $1/T_T$ the equation simplifies to:

$$\frac{1}{\text{Response}} = \frac{K_T}{k_6 M_T}\left[\frac{K_M k_{ex}}{k_3 T_T}\right]\frac{1}{Ca_o}+\frac{1}{k_6 M_T}\left[\left(\frac{K_M k_{ex}}{k_3 T_T}\right)+1\right] \qquad 24$$

According to the formulation above, the responses of a smooth muscle should vary as a rectangular hyperbolic function of the calcium ion concentration as well as the concentration of agonist. For one case at least this proposition has been tested. Measurements of the isotonic contraction

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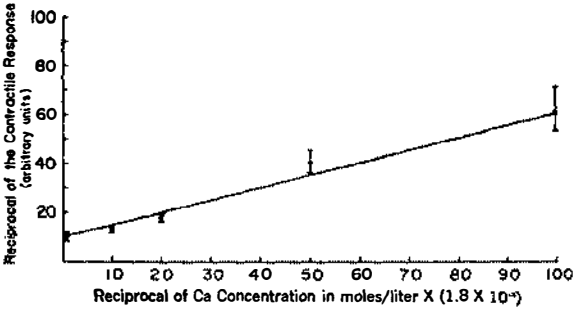

FIGURE 1. For explanation see text.

of the isolated longitudinal muscle from guinea pig ileum have been made in the presence of various extracellular concentrations of calcium ion. A potassium-rich bathing medium served as the excitatory agent. The results are shown in Figure 1, where the reciprocal of the response is plotted against the reciprocal of the calcium ion concentration. It may be seen that the data define a straight line. This indicates that the relationship between the response of the ileal muscle and the external concentration of calcium is indeed hyperbolic.

Admittedly, the series of reactions represented in the three step model and the dose-response equations derived therefrom oversimplify the situation as it actually exists in the living smooth muscle fiber. Errors in detail and in assumptions and approximations, are also difficult to avoid. Nevertheless, the model is consistent with a great many experimental findings including the inhibitory effects produced by irreversible blocking agents, the frequent occurrence of hyperbolic dose-response curves, and the essential role of calcium ions in the initiation of the contractile response. It seems reasonable, therefore to hypothesize that this model provides at least a primitive view of the manner in which the agonist is coupled to the response in smooth muscles and that, based on present knowledge, it is correct with regard to the broad conceptual image that it conveys. The fact that numerous dose-response curves do not have a hyperbolic configuration does not detract from the validity of the model. It is not difficult to see how a slight variation at any point in the series of reactions shown above (including threshold phenomena, agonist inhibition, receptor desensitization etc.) would cause a deviation from the expected shape. What is surprising is that so many dose-response curves do exhibit the hyperbolic configuration.

Perhaps the most useful aspect of the proposed model is the fact that a number of its basic characteristics are accessible to examination in the laboratory. Additional investigative work will undoubtedly help to eliminate some of the uncertainties and fill in some of the gaps that presently exist in the scheme.

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Membrane Depolarization and Response

Although the reaction pathway leading from the drug-receptor interaction to the inward transport of calcium is not well defined, some of the intermediate steps may consist of bioelectric changes in the smooth muscle membrane. In the resting muscle the fiber membrane is in a polarized state. Potential differences between the inside and outside of the smooth muscle cell range from 10-80 millivolts (55). This potential is thought to be created, in large measure, by the presence of unequal concentrations of potassium ion on the inner and outer surface of the membrane and by a high conductance of the membrane to potassium (56-61). There are indications that sodium and chloride currents also have a significant influence on the membrane potential (56-64). In addition, several workers have pointed to the possibility that an electrogenic sodium pump may be a factor in determining the magnitude of the membrane potential (59, 63, 65, 66). In spontaneously active smooth muscle fibers the membrane potential of the resting cell is not steady. Slow waves of depolarization and prepotentials induce periodic fluctuations in the membrane potential (55, 61, 67). When the polarization is reduced to a critical level an action potential or a train of action potentials is generated (55, 61, 67). It is the action potential that appears to be responsible for igniting the cellular changes that result in a smooth muscle contraction (57, 59, 61, 68). One-to-one correlations between the development of an action potential and an increase in muscle tension are a common laboratory observation.

When an excitatory drug is introduced into the extracellular environment of the muscle, the following sequence of events is thought to occur. First, the excitatory drug complexes with specific receptors in the smooth muscle membrane (1-15). This reaction presumably induces a conformational change in the receptor molecules (5, 6, 20, 29). The change in the receptor molecules brings about an increase in the permeability of the membrane to inorganic ions (20, 55, 69-72). This, in turn, reduces the magnitude of the membrane potential (55, 71, 72). As a consequence, the critical firing level of depolarization is within easier reach and the frequency and duration of trains of action potentials are increased (64, 68, 71, 73-75). In smooth muscle fibers that are not spontaneously active, the introduction of an excitatory drug will initiate the discharge of action potentials (67, 75). Under normal physiological conditions, these bioelectric changes are always accompanied by an increase in smooth muscle tension or by a shortening of the muscle fibers (64, 68, 71, 73-75).

Membrane depolarizations other than the action potential may also induce a smooth muscle contraction. A sustained level of depolarization brought about by a medium containing an isotonic concentration of potassium ion has been found to stimulate the contractile process (76-80). In some smooth muscles, the membrane change associated with a drug-induced contractile response is normally observed to be a reduction in membrane potential rather than the formation of action potentials (81-83).

Greater appreciation for the complexities inherent in the actions of smooth muscle stimulants and depressants was gained when it became evident that excitatory agents had the capacity to produce smooth muscle contractions without eliciting any changes in membrane potential. Evans & Schild (84) and Evans et al (76) were the first to demonstrate this phenomenon. They showed that a number of agonists and antagonists could enhance or inhibit the tension of smooth muscles immersed in a medium containing an isotonic concentration of K_2SO_4. Muscle fibers immersed in this bathing medium undergo appreciable membrane depolarization. The changes in tension effected by various agonists under these conditions were reported to occur in the absence of any further change in membrane potential (85). Since then, reports in the literature have revealed that epinephrine can stimulate a mechanical response in the polarized pulmonary artery (86) and the polarized stomach muscle (87) of the rabbit without invoking any reduction in membrane potential.

Based on the accumulated evidence outlined above, it would appear that agonists may stimulate contractions in smooth muscle fibers by at least two different mechanisms. One involves the generation of action potentials or, in some instances, a sustained depolarization; the other does not. In either case, it is assumed that the drug induces membrane changes that permit the entrance of calcium ions into the cytoplasm. Once in the cytoplasm the divalent ions serve to activate the contractile elements. Results of studies performed on various types of smooth muscle, but particularly vascular smooth muscle, have suggested that membrane depolarizations, whether drug-induced or not, mobilize the divalent ions primarily from the extracellular medium or from a loosely bound pool of calcium in the muscle fiber (88–94). The second mode of action of the agonist seems to affect principally the calcium ions that are sequestered or tightly bound in the muscle (88–94). Some agents seem to exert both types of actions; others only one type (89–94). In some smooth muscles, such as the longitudinal muscle of the guinea pig ileum, a tightly bound reservoir of calcium that can be mobilized for contraction appears to be lacking (95). Thus, with regard to agonist action on smooth muscle, one may observe a variety of effects depending on the agonist that is employed and on the smooth muscle preparation that is being investigated.

Calcium Transport and Response

The work done to determine the manner in which cytoplasmic calcium in smooth muscle fibers is increased has invited considerable speculation. Various proposals have been made with regard to the source of the calcium ions that enter the cytoplasm and the types of transport mechanisms that are involved.

Essentially two different reservoirs of calcium are thought to be the sources of activator ions in smooth muscle (88–94). One is the calcium that is in the extracellular medium or is loosely attached to the muscle fiber. The other is calcium that is tightly bound or sequestered in the fiber. The

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evidence that extracellular calcium ions can enter the cytoplasm and induce a contraction rests primarily in two separate but related observations. First, smooth muscle fibers exposed to a wide range of extracellular calcium ion concentrations develop drug- or potassium-induced contractions of variable magnitudes (89, 91, 96–99). When the calcium concentration is high, the contraction is large; when the calcium concentration is reduced, the contraction becomes smaller. Second, if the calcium ions in the external medium are completely removed, the fibers, whether polarized or depolarized, gradually become incapable of generating even a minimal response (49, 57, 70, 89, 95, 96, 100–111). Suspending smooth muscles in a calcium-free medium also provides a means of uncovering muscle fibers that possess a tightly bound as well as a loosely bound store of mobilizable calcium. These muscles rapidly lose their responsiveness to excitatory agents that presumably mobilize extracellular or loosely bound calcium, but remain responsive for long periods of time to agents that mobilize the tightly bound pool of calcium (89, 90, 94). Although the concept of multiple sources of activator ions in smooth muscle fibers seems to be widely accepted, some objection has been raised. Somlyo & Somlyo (83) believe that excitatory agents (high potassium, electrical stimuli, neurohormones) may differ in their actions, not because they mobilize calcium from different pools, but because they have differential effects on the permeability of the fiber membrane.

Further evidence for the roles played by extracellular and cellular calcium has been sought by following the unidirectional and net movements of calcium ion between the smooth muscle fiber and its external environment. Changes in calcium fluxes induced by agents that affect the contractile function of smooth muscles have been extensively investigated. The outcome of these studies has, however, been difficult to interpret. Excitatory agents appear to modify calcium fluxes in diverse ways depending upon the smooth muscle preparation being investigated and the experimental conditions employed. Table 1 shows, in summary form, the experimental results that have been obtained in a number of studies of this type. In view of these findings, it may be prudent to consider the possibility that the transport of activator ions into and out of the cytoplasm represents a small fraction of the total exchange of calcium that occurs between the external medium and various binding sites and depots in the smooth muscle fiber (83, 114). If this were the case, the critical calcium movements that regulate contractile function would be extremely difficult to detect.

According to some workers in the field, the influx of extracellular or loosely bound calcium into the cytoplasm of smooth muscle fibers is regulated by the permeability of the plasma membrane (83, 100, 114, 115). An agonist is assumed to modify the contractile state of a smooth muscle by inducing an increase in membrane conductance to these ions (83, 100, 114, 115). The increase in membrane conductance allows the calcium to flow down an electrochemical gradient into the cytoplasm, combine with contractile proteins, and initiate a contraction. It was thought, at one time,

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TABLE 1

Smooth Muscle Tissue	Excitatory Agent	Influx of Calcium	Efflux of Calcium	Net Change in Calcium Content	Ref.[g]
Rat Uterus	ACh[a]	0[d]	0	—[h]	88
Rat Uterus	High K^+ medium	↓[e]	0	↓	88
Depolarized Rat Uterus	ACh	↑[f]	0	—	88
Guinea pig Taenia coli	ACh	0	↑	—	112
Guinea pig Taenia coli	High K^+ medium	—	↑	—	112
Guinea pig Taenia coli	Carbachol	0	↑	—	112
Depolarized guinea pig Taenia coli	Carbachol	↑	0 or ↑ sl.[b]	—	69
Guinea pig Taenia coli	40mM K^+ medium	↑	—	↑	113
Rabbit Aorta	High K^+ medium	↑	0	0	105
Rabbit Aorta	NE[c] (Ca-free)	—	↓	—	94
Guinea pig Ileum	ACh (Ca-free)	—	0	—	109

[a] Acetylcholine.
[b] Slightly.
[c] Norepinephrine (Calcium-free medium).
[d] No change.
[e] Decreased.
[f] Increased.
[g] Reference.
[h] Not reported.

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that the influx of extracellular or surface bound calcium in striated muscle fibers served the same purpose (116, 117). However, objections to this concept soon developed. The hypothesis could not be reconciled with the fact that extracellular calcium ions diffuse into the cell too slowly to play the role of activator in the contractile process (118). Subsequent research showed that activator ions in striated muscle originate in a subcellular structure, the sarcoplasmic reticulum (119–122). This structure has been shown to be in close proximity to the contractile apparatus. Thus, the calcium ions released from the sarcoplasmic reticulum in an excited fiber, do not need to diffuse long distances to reach the elements they activate (121, 123). As a general rule, smooth muscle fibers are thinner than striated muscle fibers (124), contract more slowly (124), and lack an elaborate sarcoplasmic reticulum (124–127). Consequently, the arguments that would lead one to reject an activator role for extracellular calcium ions in striated muscle do not apply for smooth muscle.

The hypothesis that membrane permeability regulates the influx of calcium ions in smooth muscle has received support from electrophysiological studies. These studies have shown that the bioelectric potentials of a number of smooth muscles are somewhat unique. The usual underlying basis for the formation of an action potential in excitable tissues is a transitory increase in membrane permeability to sodium followed by an increase in membrane permeability to potassium (128–130). These membrane changes lead to a net influx of sodium ions producing membrane depolarization (128–130) and a subsequent net efflux of potassium ions producing membrane repolarization (128–130). In some smooth muscles that have been investigated, the configurations of the action potentials are not drastically altered by the introduction of a low sodium bathing medium (131–133), but appear to be quite sensitive to changes in the calcium ion concentration in the medium (134–138). Moreover, tetrodotoxin, a compound that specifically inhibits the permeability of the membrane to sodium ions (139, 140), proved to be without effect on the development of action potentials in these fibers (138, 141–144). Conversely, manganese ion, which has been shown to depress membrane conductance to calcium ions (145), inhibited the action potentials (135, 136, 138). These results have led to the suggestion that the action potentials in certain smooth muscles are "Ca spikes" rather than "Na spikes" (134–138). That is to say, an increase in membrane permeability to calcium rather than sodium induces the depolarization phase of the action potential.

The possible occurrence of "Ca spikes" in some smooth muscle fibers has prompted several investigators to call attention to an interesting possibility. They suggested that the divalent ions that enter the cell during a spike potential may be the ones that trigger the mechanical response (138, 146, 147). In support of this hypothesis are: (*a*) the evidence that extracellular calcium can serve as a source of activator ions, and (*b*) the observation that the development of spike potentials and tension are closely cor-

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related. In addition, it has been shown that manganese ion, an inhibitor of calcium permeability, depresses not only the spike potential but the accompanying mechanical response (138). One could argue that this effect of manganese ions on the mechanical response is an indirect one. The principal action of the inhibitory ion could be on membrane conductance to calcium and possibly other ions that are involved exclusively with membrane depolarization. However, the mechanical response of muscles that have been depolarized in a potassium-rich medium were also found to be depressed by manganese ions (115, 138). This would suggest that the inhibitory ion also affects the passive entry of calcium ions that are involved in activating the contractile apparatus. On this basis it would not be unreasonable to hypothesize that in certain smooth muscles the permeability of the fiber membrane controls the entry of calcium ions which have the dual role of depolarizing the membrane and triggering the contraction.

If the inward movement of calcium in smooth muscle is actually directed by an electrochemical gradient, the factors that determine the rate of flow are given by the Goldman equation (148). The velocity of the unidirectional movement of calcium into the cytoplasm may, therefore, be expressed as (148):

$$I = CPE_m 2F/RT(1 - e^{-E_m 2F/RT})^{-1} \qquad 25.$$

where I denotes the influx of calcium ions across the membrane, C is the concentration of calcium ions in the extracellular fluid, P is the permeability of the fiber membrane to calcium, E_m is the membrane potential and R, T, and F are the gas constant, temperature, and Faraday constant respectively.

According to the equation given above, the rate of inward movement of calcium into the muscle is directly proportional to the concentration of these divalent ions present in the extracellular fluid or in some cellular compartment. Any reduction in the rate of entry of calcium brought about by an increase in membrane resistance or by a decrease in membrane polarization, providing it is not extreme, may be compensated for by an appropriate increase in the calcium ion concentration in the bathing medium.

Studies on isolated intestinal muscle were performed to determine whether or not this equation accurately expresses the relationship that exists between the concentration of extracellular or loosely bound calcium and the rate of entry of calcium into the cytoplasm. A direct measurement of the influx of calcium into the cytoplasm of the smooth muscle fibers was not attempted. The parameter that was recorded was the magnitude of contraction of the muscle. This was assumed to be some function of the influx of calcium into the cytoplasm.

With muscles that were depolarized in a potassium-rich medium, the magnitude of the sustained contraction exhibited by the fibers increased in a hyperbolic fashion as the concentration of the calcium ions in the external medium was increased. The relationship observed is illustrated in Figure 1 where the reciprocal of the contractile response is plotted against the re-

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ciprocal of the calcium ion concentration (Hurwitz & Grijalbo, unpublished data). As mentioned previously, a hyperbolic curve becomes a straight line on a reciprocal plot. The intercept of the straight line denotes the inverse of the maximum contraction that the muscle will theoretically develop in the presence of an infinitely high concentration of calcium ions.

A similar hyperbolic relationship was observed between the contractile response and the concentration of calcium ions stored in a cellular depot (95, 109). In this case, the smooth muscles were stimulated to contract by immersing them in a calcium-free medium and the divalent ions that were mobilized for contraction came from a loosely bound pool in the fibers. A significant aspect of the study was the finding that the maximum response that would theoretically be obtained in the presence of an infinite quantity of calcium in the cellular store was smaller than the muscle was capable of developing. This finding provided a clue to the manner in which calcium ions are transported into the cytoplasm in an excited fiber. It was reasoned that if the mobilizable calcium reached the contractile proteins by diffusion through a permeable membrane in accordance with the equation shown above, then the amplitude of contraction in the presence of an infinite quantity of stored calcium should reflect the maximum capacity of the muscle to contract. Since this was not the case, one must assume that some restraint other than a simple diffusion barrier regulates the rate of movement of calcium into the cytoplasm. This restraint could conceivably be some kind of saturable transport system located in the fiber membrane (95, 109).

Edman & Schild (49) and Schild (78) working with depolarized rat uterus have obtained data which have led to a similar conclusion. They noted that the inhibitory effects of epinephrine and isoproterenol on the mechanical responses of the muscle fibers could not be reversed by increasing the extracellular calcium ion concentration. To explain the noncompetitive character of the antagonism between the catecholamines and calcium, it was suggested that the inhibitory agents may interfere with the entry of extracellular calcium into the muscle and thus prevent a sufficient buildup of free intracellular calcium. As an alternative possibility, epinephrine and isoproterenol may lower the free intracellular calcium level by modifying the operation of an energy-linked calcium-extrusion process. With regard to the latter possibility, Schild (78) pointed out that a mechanism that depends on decreasing free intracellular calcium should be swamped by very high concentrations of the divalent ion unless some barrier to the penetration of high calcium ion concentrations is present. Such a barrier would be created by a saturable transport system. On the other hand, if the barrier that limits uptake of calcium into the muscle is a saturable system, the catecholamines might also inhibit contraction in a partially noncompetitive manner by affecting inwardly directed transport.

Thus it would appear that an agonist, by interacting with its specific receptor in the target cell, may induce a conformational change in the receptor molecule that leads ultimately to: (*a*) a "Na spike potential," (*b*)

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a "Ca spike potential," (*c*) a sustained depolarization, (*d*) excitation in the absence of membrane depolarization, or (*e*) a combination of two or more of the preceding membrane reactions. The consequence of these membrane reactions is postulated to be the activation of a calcium transport system which permits extracellular or cellular calcium ions to enter the cytoplasm and trigger a contraction. The calcium transport system appears to have the characteristics of a saturable system. As indicated in the three step model presented above, the number of saturable transport sites that become operational may be related in some definite way to the number of tissue receptors occupied by a drug or, if Paton's rate theory (149, 150) is preferred, to the number of drug-receptor interactions occurring at any point in time. The concept of a definitive relationship between operational calcium transport sites and drug-receptor complexes receives support from the fact that a considerable number of dose-response curves can be described by an equation for a rectangular hyperbola.

Investigators primarily concerned with the mobilization of sequestered calcium for contraction have employed the term "release" to denote a critical step in a calcium transport process. The term is used to convey the idea that calcium ions bound in or to some cellular component are freed and thereby available to enter the cytoplasm and activate contractile elements. The release of calcium from its bound form is assumed to be the end result of a series of cellular reactions induced by an agonist. Unfortunately, little, if anything, is known about the underlying physico-chemical processes involved in the dissociation of calcium ions from its storage sites. One could speculate that the release of calcium is brought about by a change in the affinity of the divalent ions for binding sites, by a change in the retentive capacity of some vesicular structure, or perhaps by some other mechanism. Based on studies of the inhibitory effects of local anesthetics on the contractile function of smooth muscle and on the binding of calcium to various molecules, Feinstein & Paimre (151) dismissed the hypothesis that these agents act by decreasing the dissociation of calcium from molecular binding sites. They suggested, instead, that the local anesthetics inhibit the release of calcium from a cellular depot by reducing the membrane permeability of an intracellular organelle that sequesters calcium. In any case, the term "release" currently represents a poorly understood drug-induced cellular reaction which makes possible the translocation of calcium from a cellular depot, where the divalent ions are sequestered, to the cytoplasm.

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