CHAPTER 3

Ca^{2+} and Respiratory Smooth Muscle Function

Is There a Role for Calcium Entry Blockers in Asthma Therapy?

Jeffrey S. Fedan, Douglas W.P. Hay, and David Raeburn

The purpose of this chapter is to discuss the actions of Ca^{2+} entry blocking drugs (CEBs)—also variously referred to as Ca^{2+} antagonists, Ca^{2+} channel blockers, and Ca^{2+} channel inhibitors—on the respiratory smooth muscle of experimental animals and humans in vitro and in vivo. An overview of the role of Ca^{2+} in excitation–contraction coupling mechanisms in respiratory smooth muscle will first be presented, as research in this area is only recently developing, and there are some differences between the effects of CEBs on airways and other smooth muscles. In light of recent interest in CEBs as drugs for treating bronchoconstrictive disorders, the potential therapeutic role of this class of drugs in the treatment and/or prophylaxis of asthma and other respiratory disorders will be discussed.

Ca^{2+}, CEBS, AND AIRWAY SMOOTH MUSCLE CONTRACTION

Pharmacomechanical and Electromechanical Coupling

Compared to vascular, intestinal, and other smooth muscles, excitation–contraction coupling and the actions of CEBs in airway smooth muscle have not been reviewed extensively. The information available (for recent reviews see refs. 1–4) indicates that, in general, the excitation–contraction coupling mechanisms in airways and nonairway smooth muscle share many similarities. Thus, both electromechanical and pharmacomechanical coupling[5,6]

may be utilized to elicit a mechanical response. The contraction of airway smooth muscle depends on an increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). This increase may be brought about in various ways. Agents such as KCl that produce a graded depolarization of the smooth muscle cell membrane cause the opening of voltage-sensitive or voltage-dependent Ca^{2+} channels (VDCs) in the cell membrane. When the channel opens extracellular Ca^{2+} enters the cell down its concentration gradient. This mechanism is referred to as *electromechanical coupling*. On the other hand, the interactions of agonists, for example acetylcholine and histamine, with their respective receptors can induce the opening of another type of membrane Ca^{2+} channel, the receptor-operated Ca^{2+} channel (ROC) independent of membrane depolarization. This mechanism is referred to as *pharmacomechanical coupling*. In addition, small amounts of Ca^{2+} ("trigger Ca^{2+}") can enter the cell and induce the release of intracellular Ca^{2+}, a process often called *Ca^{2+}-induced Ca^{2+} release*.

Only a few studies on the roles of the two coupling mechanisms in airway smooth muscle have been published; electrophysiologic studies are especially lacking. The relative importance of electromechanical versus pharmacologic coupling appears to be related to the concentration of excitatory agent.[7-9] At low agonist concentrations responses are initiated by electromechanical coupling, but as the agonist concentration increases, pharmacomechanical coupling becomes the predominant mechanism mediating the contraction. For example, Farley and Miles[7,8] observed that in concentrations ≤ 1 μM, acetylcholine-induced contractions of dog trachea involve membrane depolarization and Ca^{2+} influx via membrane Ca^{2+} channels that are blocked by verapamil; at higher concentrations of acetylcholine the contraction was independent of changes in the membrane potential, was relatively insensitive to verapamil, and was due possibly to the mobilization of intracellular Ca^{2+} or tightly bound extracellular Ca^{2+}. Somewhat different results were observed by Coburn,[9] who did not observe in dog trachealis muscle an uncoupling of the relationship between acetylcholine concentration and membrane depolarization. In airway smooth muscle, as in other smooth muscles, generalities regarding the importance of electromechanical coupling at low agonist concentrations and of pharmacomechanical coupling at high agonist concentrations do not appear to apply. For example, in rabbit trachea a high concentration of acetylcholine (8.9 μM), but not a lower concentration (8.9 nM), stimulated ^{45}Ca uptake via a gallopamil-sensitive pathway.[10] Such species differences in mechanisms of contraction in homologous tissues are discussed elsewhere in this chapter.

Sources of Ca^{2+} for Contraction

In airway smooth muscle, contraction resulting from Ca^{2+} influx through VDCs under conditions in which the membrane is depolarized (as with KCl)

appears to be more easily inhibited by CEBs than responses occurring after receptor activation. A possible explanation for this differing effectiveness of CEBs is that, in addition to ROCs showing a lower sensitivity to CEBs,[6] agonist–receptor interactions cause the mobilization of Ca^{2+} from an internal store(s). The relative dependence of responses of intra- and extracellular Ca^{2+} is not the same for all excitatory agonists. Thus, variations in the sensitivity of responses to antagonism by CEBs will depend on the particular agonist and its concentration.

Receptor-mediated responses of airway smooth muscle to a variety of agonists, while not clearly biphasic in an overt sense, are ordinarily comprised of an initial fast component and a secondary slower phase. The two phases have been suggested to reflect the utilization of different sources of Ca^{2+}. "Initiation of the response may depend upon release of Ca^{2+} from an intracellular store, whereas the secondary, sustained phase relies on the influx of extracellular Ca^{2+} to maintain an elevated $[Ca^{2+}]_i$.

In smooth muscle, contraction is initiated following the phosphorylation of P-light chains of myosin by myosin light chain kinase.[12] This enzyme depends on and is activated by Ca^{2+}·calmodulin. Phosphorylation of the myosin light chains results in actomyosin ATPase activity, cross-bridge cycling, and contraction.[13] Myosin light chain phosphorylation and cross-bridge cycling are transient events which follow in close association a rapid and transient elevation of $[Ca^{2+}]_i$. As the degree of light chain phosphorylation and the $[Ca^{2+}]_i$ decline, a second mechanism is brought into play, in which attached cycling cross bridges are converted via an unknown mechanism into noncycling cross bridges, which have been referred to as *latch bridges*.[14] The maintenance of a sustained level of force by latch bridges relies on extracellular Ca^{2+}—removal of the cation from the extracellular fluid results in relaxation. An important feature of this mechanism is that the force maintained by latch bridges requires a substantially lower $[Ca^{2+}]_i$ than the concentration found on initiation of the contraction, ie, when cross bridges are cycling. Low levels of Ca^{2+} influx may therefore provide sufficient $[Ca^{2+}]_i$ to maintain the latch-bridge mechanism during the second phase of the response. The effects of CEBs on smooth muscle must now be evaluated in the light of the knowledge that both intracellular and extracellular Ca^{2+}, and both cycling and noncycling cross bridges, are involved in receptor-mediated responses. Accordingly, the ability of CEBs to prevent a contraction (an antispasmogenic effect) and to ablate an already developed contraction (a spasmolytic effect) would be predicted to be different.[15] This important theme will be expanded throughout the chapter.

A confounding consideration relevant to the use of CEBs to evaluate the role of Ca^{2+} in smooth muscle function and whether responses are pharmacomechanically or electromechanically coupled, is that the three most often used types of organic CEBs—verapamil, diltiazem, and 1,4-dihydropyri-

dines such as nifedipine—are not pharmacologically equivalent. While the issue is not resolved, the first two classes of compounds alter Ca^{2+} channel kinetics and have, especially in cardiac muscle, a "use-dependence"; the dihydropyridines appear not to affect channel kinetics to the same extent, and may bind to the channel in its inactivated state following membrane depolarization.[16] Moreover, the three CEBs bind to different sites on Ca^{2+} channels.[17]

Broadly speaking, the potential value of CEBs in the treatment and/or prophylaxis of asthma is being examined using several approaches: (1) the prevention or reversal of experimentally induced bronchospasm in laboratory animals in vitro and in vivo; (2) the antagonism of responses to bronchoconstrictor agents in vitro of human tissue removed during surgery or at autopsy; and (3) the ability of CEBs to prevent or reverse in vivo bronchospasm induced by various pharmacologic stimuli in asthmatic and nonasthmatic humans.

Differences in Large and Small Airways

The morphologic, physiologic, and pharmacologic characteristics of the ways vary considerably along the mammalian respiratory tract. For example, the density and distribution of excitatory and inhibitory innervation and the numbers and, in some cases, the types of smooth muscle receptors exhibit marked variation from the large and central to the peripheral airways.[18-23] There are therefore qualitative and quantitative regional differences in neurogenic responses, and in responses to agents that cause contraction or relaxation. The electrophysiological properties of airway smooth muscle cells varies topographically.[24] There are regional differences in the distribution of various specialized cell types, for example, mucus-secreting cells, submucosal glands, and goblet cells.[25,26] The number of mast cells increases as the airway diameter decreases.[27,28]

Recent evidence indicates that cartilage may be a source of Ca^{2+} that is utilized in contractile responses of airway smooth muscle (see below). Regional variations in the abundance of cartilage may be physiologically important, as cartilage decreases in abundance progressively from the trachea to the distal airways and is absent in the bronchioles and alveoli.[29]

There is increasing evidence for a modulatory role of the epithelium in controlling airway smooth muscle reactivity.[30-34] In this regard the morphology of the epithelium and the number of different cell types varies considerably in the different regions of the lung.[35] In rabbit airways there are regional variations in the magnitude of the modulatory influence of the epithelium.[33]

The mechanisms of smooth muscle excitation–contraction coupling may exhibit regional and age-related variations. These variations could be manifest as, or be due to:

1. Differences in the relative utilization of extracellular and intracellular Ca^{2+} by bronchoconstrictor agents
2. Differences in the electrophysiological properties of the smooth muscle cells or in the relative involvement of electromechanical and pharmacomechanical coupling
3. Variations in the relative cellular content of sarcoplasmic reticulum,[36,37] in the size of intracellular Ca^{2+} stores, and in the efficiency of Ca^{2+} transport and release mechanisms
4. Differences in the density and the electrophysiological and/or biophysical characteristics of the smooth muscle membrane Ca^{2+} channels (for example in their gating properties affinities for CEBs) which could contribute to regional differences in the potency of CEBs.
5. Differences in the activation of the phosphatidylinositol pathway in response to agonists.

There is some evidence to suggest that some regional variations in excitation–contraction coupling mechanisms do exist. Ba^{2+}-induced contractions of guinea pig bronchi were more resistant to inhibition by nitrendipine or verapamil than were responses of the trachea, whereas nitrendipine had no effect on histamine- or carbachol-induced responses at either level in the airways.[38] In contrast, the effectiveness of verapamil in inhibiting KCl-, histamine- and methacholine-induced contractions of rabbit airway smooth muscle increased as the airway diameter decreased.[33]

The actions of nitrendipine and verapamil in tissues from young guinea pigs were reversed by washing, whereas their effects in airways from older animals were irreversible.[38] This result suggests there are age-related differences in the properties of the Ca^{2+} channel and in its affinity for CEBs. Furthermore, contractions in airway preparations from young guinea pigs were more sensitive to CEBs and to reductions in extracellular Ca^{2+} than responses of tissues from older animals.[38] It is worth considering whether these changes are related to the calcification of cartilage with age[29] (see also Cartilage as a Source of Extracellular Ca^{2+}, below).

An examination of the relative sensitivities of contractile responses to exogenous Ca^{2+} at several levels of dog airways, obtained using tissues placed in a K^+-depolarizing solution, showed that the spectrum of sensitivity to Ca^{2+} (greatest to least) was fourth generation > third generation > trachea.[39]

The contractile response of guinea pig trachea to leukotriene D_4 (LTD_4) was abolished following prolonged incubation of tissues in a Ca^{2+}-free medium, whereas the maximum response of parenchymal strips was reduced by only approximately 50%.[40] This result may reflect differences in the relative utilization of extracellular Ca^{2+} by these preparations. However, the results

may also indicate differences in the utilization of intracellular Ca^{2+}, as responses to LTD_4 of both tracheal and parenchymal strips were not affected greatly by nifedipine, but were abolished by 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8), the putative intracellular Ca^{2+} antagonist.[41] No difference was found in the effects of verapamil on LTD_4-induced contractions of rhesus monkey trachea and lung parenchyma.[42] Small differences in the effects of reduced extracellular Ca^{2+} on responses to LTC_4 and arachidonic acid were observed in guinea pig trachea and lung parenchyma, although regional differences in the effects of CEBs were not evident.[43] In contrast, Burka[44] reported differences in the ability of verapamil to inhibit LTD_4- and histamine-induced contractions of guinea pig trachea and lung parenchyma. The high concentration of verapamil (300 μM) used in that study, however, makes it difficult to draw conclusions concerning possible differences in Ca^{2+} translocation mechanisms in these preparations. Last, antigen-induced responses of guinea pig lung parenchyma were more resistant than those of trachea to Ca^{2+} deprivation[44,45] and CEBs.[44,46]

Most studies on the pharmacology and physiology of respiratory smooth muscle have employed large and central airways. The reason is largely a practical one, related to the ease of dissection and preparation of the tissue. Since its introduction,[47] the isolated lung parenchymal strip has been employed to investigate the reactivity of small airways. However, the use of this preparation is open to criticism. Unlike the large airways, from which reasonably homogeneous preparations containing primarily smooth muscle can be prepared, the lung parenchymal strip consists of at least three major components that have potential contractile activity—airway smooth muscle, vascular smooth muscle, and alveolar interstitial cells.[48–52] Responses of lung parenchymal strips to bronchoactive agents are the net result of the effect on the different cellular types. A degree of randomness in the geometric orientation of contractile cells with respect to the plane of measurement is unavoidable. There is quantitative and qualitative variability between individual preparations.[51–54] Using ultrathin preparations of guinea pig lung parenchyma that contained no conducting airways or blood vessels, Drazen and Schneider[49] concluded that contractions produced by histamine and carbachol were mediated by alveolar interstitial cells. While this is itself important information, it indicates that extrapolating results from the parenchymal strip as a means of understanding the characteristics of the smooth muscle of small airways must be done with a realization of the limitations.

The occurrence of regional differences in excitation–contraction mechanisms and the use of different diameter airways in experiments are not merely of theoretical interest. The design of experiments which are likely to be clinically relevant to the understanding of asthma and other respiratory disorders should take into account the role of the different diameter airways

in the disease. For example, in healthy individuals it appears that the greatest airway resistance occurs in airways of >2 mm inside diameter.[55,56] There is considerable evidence implicating the larger airways in acute airway obstruction.[53,55,56] A number of researchers have, however, suggested that even during the initial pathologic changes in asthma, a significant degree of narrowing occurs in the peripheral airways.[53,55–59] In chronic obstructive lung disorders such as emphysema and chronic bronchitis, increased airway resistance appears to be located primarily in the smaller airways.[53,55] It has been suggested that a similar situation exists in asthmatic patients who are smokers, in patients with bronchitis, and in patients who have recurrent infections of the respiratory tract. In asthmatic patients without these confounding factors, it was suggested that constriction of the larger airways is responsible for the increase in airway resistance.[60] The relative involvement of large and small airways in asthma may well vary between and within individuals. The substantial variability in the effects of CEBs on pulmonary function in normal and asthmatic patients could reflect the diversity of such bronchoconstrictive changes in the lung.

Studies on Animal Isolated Airways

Ca^{2+}-Dependence of Contractile Responses versus the Pharmacological Effects of CEBs: Antispasmogenic and Spasmolytic Actions

Compared to studies on the antispasmogenic effects of CEBs, there have been relatively few studies on their spasmolytic properties. Few direct comparisons of these two aspects of CEB action have been performed. The purpose of this section is to examine results relating to the spasmolytic properties of CEBs in airway smooth muscle. In the following sections of this chapter the antispasmogenic actions of CEBs will be emphasized because this aspect of their effects constitutes the bulk of the information available.

Probably the first direct comparison of antispasmogenic and spasmolytic effects of CEBs was by Cheng and Townley.[61] In studies using guinea pigs and rats, concentrations of nifedipine up to 1 μM had little antagonistic effect on histamine-induced contraction of trachealis muscle. However, this CEB was a potent spasmolytic in tissues contracted by histamine. At the highest concentration of histamine used (100 μM), nifedipine had an IC_{50} value of approximately 1 μM, whereas at the lowest histamine concentration (10μM) the nifedipine IC_{50} was approximately 10 nM, Verapamil, while not as potent as nifedipine, also exhibited spasmolytic activity. The antispasmogenic action of verapamil was not reported for comparison. Since this first investigation, the antispasmogenic and spasmolytic effects of CEBs on con-

tractile responses produced by agonists and by KCl, tetraethylammonium (TEA), and Ca^{2+} have been compared in more detail.

Guinea pig trachealis contracted by acetylcholine and histamine was relaxed by concentrations of nifedipine that had no antispasmogenic activity.[15] In contrast, the antispasmogenic and spasmolytic effects of nifedipine in tissues challenged with KCl, TEA, and Ca^{2+} (in a depolarizing medium) were comparable. A similarity has also been observed between the antispasmogenic and spasmolytic effects of verapamil on KCl-induced contractions.[62]

We have compared the antispasmogenic and spasmolytic properties of verapamil, gallopamil, diltiazem, and nifedipine on contractions produced by high (3 mM) and low (0.3 mM) concentrations of Ca^{2+} in depolarized trachealis muscle from guinea pigs. Although preliminary, the results suggest that at low Ca^{2+} concentrations there are no significant differences between the antispasmogenic and spasmolytic actions of any of the CEBs examined. However, at the higher Ca^{2+} concentration, gallopamil and verapamil had greater spasmolytic than antispasmogenic activity. The reason for this difference is not clear at present.

In the broadest terms, CEBs appear to have the expected antagonistic effects only under conditions where the influx of extracellular Ca^{2+} appears to provide the Ca^{2+} for contraction — ie, the monophasic responses to KCl, TEA, and Ca^{2+}, and the sustained secondary phase (latch-bridge mediated) of receptor-induced responses.

The relative involvement of extracellular, membrane bound, and intracellular Ca^{2+} in force generation depends on a number of factors, including the particular agonist, the agonist concentration, the specific smooth muscle tissue, and the species from which it is obtained.

The qualitative alterations in the shape of contractile responses to agonists obtained after reducing the extracellular Ca^{2+} concentration provides information on the sources of Ca^{2+} for the initial and secondary phase of the response.[62-64] The initial and secondary phases are differently affected by removal of extracellular Ca^{2+}. Responses induced by histamine, methacholine, acetylcholine, and LTD_4 obtained in Ca^{2+}-free media resolve into two distinct phases. The first phase is relatively unaffected by reducing extracellular Ca^{2+}, whereas the second, sustained component is preferentially depressed. With increasing time in the Ca^{2+}-free medium the separation into phasic and tonic components becomes even more evident (Fig. 3.1). The second phase is eventually abolished, whereas the initial phase, although depressed, remains even after a considerable time in Ca^{2+}-free medium. The effect of Ca^{2+} removal on responses induced by KCl or TEA is quite different. Here, a time-dependent and progressive reduction in response magnitude occurs, and responses remain apparently monophasic.[62] Thus, the entire response produced by KCl and TEA is dependent on Ca^{2+} influx.

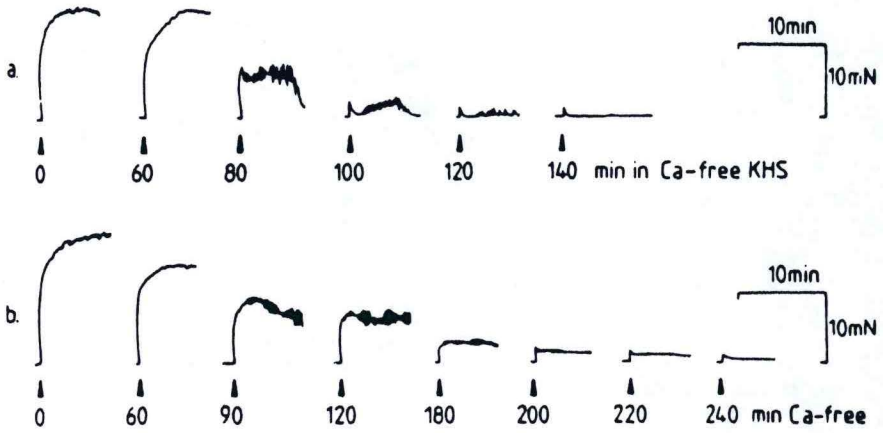

FIGURE 3.1 Effect of reducing the extracellular Ca^{2+} concentration on agonist-induced responses of guinea pig isolated trachealis preparations. After the control response was obtained in modified Krebs-Henseleit solution containing 2.5 mM Ca^{2+} ($t = 0$), the tissues were incubated continuously in nominally Ca^{2+}-free solution and repetitively challenged at the times indicated. At the arrows the tissues were exposed to histamine (40 μM; panel A) or methacholine (100 μM; panel B). These concentrations approximate the EC_{90} values. It can be seen in each case that solution the initial (phasic) component remained even after prolonged incubation in low-Ca^{2+}, whereas the sustained (tonic) component was progressively and preferentially depressed.

These results suggest that the initial phase of contraction after receptor activation involves the mobilization of a source of Ca^{2+} that is not readily affected by reducing the extracellular Ca^{2+} concentration. Based on these findings, it is generally held that intracellular Ca^{2+} is used for the initial phase of the response to agonists. In contrast, the tonic phase of the response, which it is suggested is maintained via the latch-bridge mechanism, depends on the influx of extracellular Ca^{2+}. It is probable, therefore, that for agonists the greater spasmolytic than antispasmogenic effect of CEBs is due to their inhibition of Ca^{2+} influx during the sustained phase. On the other hand, the magnitudes of the antispasmogenic and spasmolytic effects of CEBs on KCl-induced contractions are quite similar,[15,62] as would be expected for an agent that relies predominantly on extracellular Ca^{2+}.

As implied above, most studies on mammalian airway smooth muscle indicate that responses produced by agents with a physiologic role are markedly less dependent upon the influx of extracellular Ca^{2+} than contractions induced by nonphysiologic agents. For example, responses produced by KCl, Ba^{2+}, and TEA are more easily inhibited by incubation in Ca^{2+}-free physiologic solutions or by CEBs than those produced by muscarinic, histamine, and leukotriene agonists.[8,15,42,62,65-72] Additional evidence to support the tacit generalization that physiologic and nonphysiologic excitatory

agents elicit contractions that involve different Ca^{2+} translocation mechanisms and sources was provided with the 1,4-dihydropyridine agonist compound BAY K 8644, which activates voltage-dependent Ca^{2+} channels.[73] This compound potentiated KCl- and TEA-induced contractions of guinea pig trachea, whereas those induced by histamine or acetylcholine were unaffected.[74]

Sr^{2+} inhibits Ca^{2+} accumulation and binding at high-affinity (possibly intracellular) sites, but does not interfere with low-affinity (extracellular or superficial?) binding sites.[75] Contractions of tracheal smooth muscle produced by cholinergic agonists, histamine, and LTD_4 were inhibited to a greater extent by Sr^{2+} than those induced by KCl.[10,62] Given that contractions induced by KCl show a greater dependence on extracellular Ca^{2+} than those mediated via receptor activation, it would appear that there exists a direct relationship between the dependence of an agonist on extracellular Ca^{2+} and the ability of Sr^{2+} to act as a substitute for Ca^{2+} and support contractile responses induced by that agonist.

However, the evidence that the physiologically relevant agonists mobilize primarily intracellular Ca^{2+} to produce contraction is equivocal. For example, contractions induced by leukotrienes in the guinea pig trachea were reported to depend on extracellular Ca^{2+}, but they were insensitive to nifedipine, verapamil, and diltiazem.[40,72,76,77] Creese and Denborough[78] concluded that KCl-induced contractions of guinea pig trachea are *less* dependent on extracellular Ca^{2+} than those produced by histamine. In guinea pig lung parenchymal strips it was reported that carbachol and KCl use mainly extracellular Ca^{2+}, whereas histamine uses both extracellular and intracellular Ca^{2+}.[45] Furthermore, similar reductions in force development after incubation in a Ca^{2+}-free physiologic solution or in the presence of La^{3+} were reported for KCl, histamine, and acetylcholine in guinea pig trachea.[62,65] In addition, despite the sensitivity of responses to removal of extracellular Ca^{2+}, histamine- and carbachol-induced contractions of guinea pig trachea and bronchi were unaffected by nitrendipine or verapamil.[38]

A possible reason for the above discrepancies is that different protocols were used. Differences include the length of incubation in Ca^{2+}-free physiologic solutions, the absence or presence and concentrations of the Ca^{2+} chelators ethylenediamine-*N,N,N'*,*N'*-tetraacetic acid (EDTA) and ethyleneglycol-*bis*-(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), the concentrations of the CEBs, and the equilibration periods employed. For example, incubation of guinea pig trachea for 1 hour in Ca^{2+}-free medium containing 0.1 mM EGTA abolished acetylcholine- and LTD_4-induced contractions.[40] However, as was acknowledged in the report, such a prolonged incubation period is likely to deplete intracellular Ca^{2+} stores because of leakage across the cell membrane and subsequent chelation of Ca^{2+}. Some

studies[45,67,78,79] have involved the use of EGTA or EDTA in high concentrations (2–3 mM) that are likely to damage the integrity of the smooth muscle cell membrane.[80] Furthermore, CEBs have been used in concentrations ranging from 1 nM to ≥ 100 μM, and at the higher concentrations could exert one or more of a spectrum of nonspecific effects (see Nonspecific Actions of CEBs, below).

The disparities among the aforementioned results and conclusions highlight the fact that caution should be used in interpreting the effect of extracellular Ca^{2+} removal. For example, a response that is abolished in a Ca^{2+}-free medium may require the influx through ROCs of a small amount of extracellular "trigger" Ca^{2+}, which subsequently releases Ca^{2+} from intracellular sites.[6] Accordingly, responses that are sensitive to removal of extracellular Ca^{2+} may nevertheless use predominantly intracellularly released Ca^{2+} and be largely unaffected by CEBs. A response may be initiated primarily by the release of intracellular Ca^{2+} but still require the influx of extracellular Ca^{2+} to maintain the contraction or to replenish the intracellular Ca^{2+} stores.[15,40,78] Contractions not abolished in Ca^{2+}-free solution may be mediated, at least in part, by entry of tightly bound extracellular Ca^{2+} which is not removed completely by incubation in a Ca^{2+}-free medium. Recently it has been shown that superfusion of guinea pig trachea with Ca^{2+}-free medium does not remove all the extracellularly bound Ca^{2+}.[81]

In summary, most of the evidence indicates that endogenous agonists use predominantly Ca^{2+} released from intracellular stores. Accordingly, assuming that at least some of these agents have a role in the etiology of asthma, it is not surprising that the CEBs have limited clinical efficacy in this disease.

Species Differences in Ca^{2+} Handling

A number of species differences in sensitivity to exogenous Ca^{2+}, in the role of extracellular Ca^{2+}, and in the effects of CEBs in airway smooth muscle have been reported.

In tissues depolarized with elevated extracellular K^+, tracheal preparations from guinea pigs were significantly more sensitive to Ca^{2+} than were dog large and small airways, as assessed by a comparison of EC_{50} values.[39] However, no species difference was apparent in the potencies of verapamil, nifedipine, or diltiazem as antagonists of responses to Ca^{2+}. This suggests that the reason for the species difference in sensitivity to Ca^{2+} involves a site other than the antagonist binding sites on the Ca^{2+} channel, ie, in the contractile proteins or Ca^{2+} efflux and/or sequestration systems.

Sr^{2+} as a substitute for Ca^{2+} was more effective in maintaining KCl-induced contractions of guinea pig trachea (unpublished observations) than of

rabbit trachea.[10] Creese and Denborough[78] concluded that histamine- and acetylcholine-induced responses of guinea pig trachea were more sensitive to the removal of extracellular Ca^{2+} (in the presence of 2.5 mM EGTA) than were responses of bovine tracheal smooth muscle to these agents (in the presence of 0.1 mM EGTA).[66,82] However, for the reasons outlined above, the higher EGTA concentration (2.5 mM) and the longer incubation period used in the study by Creese and Denborough[78] cast doubt on whether species difference actually exist. Lastly, LTD_4-induced contractions of rhesus monkey trachea[42] were more sensitive to verapamil than were those of guinea pig trachea.[40,44,62]

Ovalbumin-Sensitized Guinea Pigs

A hypothesis of the etiology of asthma and other hyperreactive airway disorders is that bronchoconstriction is brought about by a cellular defect in Ca^{2+} handling which results in an elevation of $[Ca^{2+}]_i$ (see for example ref. 83). Evidence to support this hypothesis was provided a few years ago when it was reported that trachea from ovalbumin-sensitized guinea pigs exhibited an increased sensitivity to extracellular Ca^{2+} compared with tissues from control animals.[84,85] The ovalbumin-sensitized guinea pig is used as a model of airway hyperreactivity.[86-88] In addition, the mobilization and binding of Ca^{2+} involved in histamine-induced contractions was altered in in vitro tracheal preparations from ovalbumin-sensitized guinea pigs.[62,89] There has been no further publication of similar studies, perhaps because of the inherent limitations of the model. Several groups of researchers have shown that ovalbumin sensitization of guinea pigs does *not* result in airway hyperreactivity in vitro.[90,91] In fact, the sensitivity to histamine and methacholine of airways from sensitized animals is decreased (see ref. 92 and references inter alia). Both the basal level and the KCl-stimulated uptake of ^{45}Ca are the same in tracheal smooth muscle from control and ovalbumin-sensitized guinea pigs.[93] This would suggest that the increased sensitivity to Ca^{2+} of airways from sensitized animals observed previously[84,85] is not due to an increase in the entry of Ca^{2+}, but rather that it may be mediated by an alteration(s) in Ca^{2+} handling mechanisms beyond the level of the cell membrane and Ca^{2+} channels.

Addition of ovalbumin to isolated airways from ovalbumin-sensitized guinea pigs produces a contraction resulting from an antigen/antibody reaction on mast cells[94-96] and the release of bronchoactive mediators (see ref. 92 and references inter alia). The antigen-induced contraction of guinea pig trachea was abolished (unpublished observations) or markedly depressed[44] after incubation in a Ca^{2+}-free medium containing or lacking EGTA. However, CEBs have been reported to produce contrasting effects on antigen-induced contractions: no effect,[97] and inhibition at high[44] or relatively low[98]

concentrations. In these experiments the mast cell may be the alternate target for the inhibitory effects of Ca^{2+} removal and the actions of CEBs on responses to antigen challenge (see Inhibition of Mediator Release, below).

^{45}Ca Flux Studies

A limited number of ^{45}Ca flux studies have been performed using airway smooth muscle. In the first report it was observed that in the guinea pig trachealis TEA produced an approximately twofold increase in La^{3+}-resistant ^{45}Ca uptake above basal levels, which was in agreement with the finding that contractile responses to TEA depended on the influx of extracellular Ca^{2+}.[99] KCl was later shown to also stimulate ^{45}Ca uptake into guinea pig trachealis muscle[65,72,100] via a verapamil-sensitive pathway.[72,100] In contrast, acetylcholine, methacholine, and LTD_4, which are thought to use primarily intracellular Ca^{2+}, did not increase La^{3+}-resistant ^{45}Ca uptake.[11,62,72,100] On its own this finding would suggest that contractile responses induced by these three agents are mediated entirely by the release of intracellular Ca^{2+}. However, this conclusion is not in agreement with tension studies which indicate that a portion of acetylcholine-, histamine-, and LTD_4-induced contraction is inhibited by removal of extracellular Ca^{2+} and by CEBs (see above). The different findings may reconciled if it is remembered that the small amount of extracellular Ca^{2+} that acts as trigger Ca^{2+} or that otherwise maintains force development (for example, by latch bridge maintenance), may not be detectable with the "La^{3+} method." Further evidence that LTD_4 uses an intracellular Ca^{2+} store for contraction of guinea pig trachea was provided in a recent study in which LTD_4 (but not KCl or TEA) increased ^{45}Ca efflux following treatment of the tissue with La^{3+} to remove all extracellularly bound Ca^{2+}.[81] A discrepancy in the generalization that it is difficult to demonstrate a La^{3+}-resistant, agonist-stimulated ^{45}Ca uptake is derived from studies on histamine action. Unlike Ahmed et al,[11] Raeburn[62] observed a small but significant histamine-induced increase in ^{45}Ca uptake in guinea pig trachea, which appears to originate from an extracellular source of Ca^{2+} different from that mobilized by KCl.[72]

Cartilage as a Source of Extracellular Ca^{2+}

In ^{45}Ca uptake experiments on guinea pig trachea the basal cellular Ca^{2+} content of preparations containing cartilage was approximately threefold higher than in preparations from which the cartilage had been removed.[11,65,72,99] Furthermore, KCl and TEA increased ^{45}Ca uptake in tracheal preparations lacking cartilage but not in those containing cartilage.[65,72,99] In fact, KCl decreased ^{45}Ca uptake in the presence of cartilage.[65,72] Thus, airway cartilage appears to be a site of substantial Ca^{2+} binding, and there is an interaction between the binding of Ca^{2+} and K^+.

The question arises as to whether this cartilage-bound Ca^{2+} is involved in airway smooth muscle contraction. Agonist-induced responses of guinea pig and rabbit tracheal preparations lacking cartilage were more strongly reduced when incubated in a Ca^{2+}-free medium than were contractions of intact preparations; this effect was especially pronounced in guinea pig trachea.[101] In view of the common embryologic origin of intestinal and respiratory tissues, it is of interest that the dependence on extracellular Ca^{2+} of methacholine-, histamine-, and K^+-induced responses of cartilage-free guinea pig tracheal preparations resembles those obtained in taenia coli.[101] In the absence of La^{3+}, KCl and TEA increased ^{45}Ca efflux from guinea pig tracheal preparations lacking cartilage.[81] Conversely, it has been reported recently that KCl increased ^{45}Ca efflux from the cartilage but not the smooth muscle of guinea pig trachea.[102] The conclusion to be drawn from the above studies is that cartilage appears to be a source of Ca^{2+} that can be used for airway smooth muscle contraction. A point for consideration is whether this cartilage-bound reservoir of mobilizable extracellular Ca^{2+} exerts any influence on the potency of CEBs, and whether it contributes to the relative ineffectiveness of these agents in asthma.[101]

The amount of Ca^{2+} derived from proximate cartilage could possibly be modified by aging and by airway diseases if they affect the binding of Ca^{2+} to cartilage. For example, cartilage becomes calcified with advancing age.[29] The amount of cartilage in the walls of the airways decreases progressively from the trachea to smaller-diameter airways.[29] There might then exist an inverse relationship between the Ca^{2+}-dependence of responses and the sensitivity of these responses to CEBs on the one hand, and airway diameter on the other. The fact that the sensitivity to Ca^{2+} in K^+-depolarized airway preparations from dogs increases with decreasing airway diameter[39] suggests that this may be indeed the case.

Role of the Epithelium in Airway Smooth Muscle Reactivity and Its Influence on the Effects of CEBs

Loss or damage of epithelial cells are pathologic features of asthma and other respiratory disorders which, in vivo, accompany increased airway responsiveness to bronchoconstrictor agents.[103,104] It has been shown in vitro that mechanical removal of the epithelium increases the sensitivity to a variety of bronchoactive agents and to neurogenic stimulation of large and central airways from various species including guinea pigs,[30,34] dogs,[32] rabbits,[33] cows,[31] and humans.[105] In addition, contractile responses of guinea pig preparations from sensitized animals to antigen also were potentiated following epithelium removal.[92] These studies provide evidence that an epithelium-derived inhibitory factor or factors modulates the reactivity of airway smooth muscle. The increased reactivity produced by removal of the epithelium does not appear to be due to alterations in extracellular Ca^{2+} influx.[93]

The inhibitory effects of verapamil on methacholine-, histamine-, and KCl-induced responses were decreased in epithelium-free preparations of several levels of rabbit airways.[33] This result suggests that the epithelium is a determinant of the activity of CEBs. The mechanism by which this effect occurs is not known. Two further effects of epithelial cell loss and damage in airway hyperreactivity diseases may be (1) a reduction in the influence of a epithelium-derived inhibitory factor(s), which may increase the bronchoconstriction produced by endogenous stimulants; and (2) a reduction in the ability of CEBs to antagonize contractile responses to such agents. It is possible that the release of the epithelium-derived factor(s) is Ca^{2+}-dependent, in the way described for the vascular endothelium-derived relaxing factor.[106] The modulatory role of the epithelium on the effect of CEBs in airways seems worth further pursuit.

Effects of CEBs and Reduced Extracellular Ca^{2+} on Basal Tone

The airways of some mammals develop spontaneous tone in vitro. There are conflicting reports on the effects of CEBs on this phenomenon. Most studies in guinea pigs indicate that CEBs exert no effect.[15,38,40,62,72,97] This is in agreement with in vivo studies in which the CEBs appear not to affect basal pulmonary function (see In Vivo Studies, below). In contrast, verapamil and nifedipine reduced basal tension of the dog trachea in situ.[107] Nifedipine (30 nM) has been reported to cause relaxation of the basal tone of guinea pig trachea and parenchyma in vitro; nevertheless, it had no effect on pulmonary mechanics in vivo.[108] A relaxant effect of nifedipine on basal tone of guinea pig trachea was reported by Cheng and Townley,[61] and diltiazem and verapamil also were reported to affect the basal tone of this tissue, but only in very high concentrations ($\geq$100 μM).[67]

Studies of the effect on basal tone of removing extracellular Ca^{2+} have yielded conflicting results. In most cases a substantial reduction in the resting tone of guinea pig trachea was seen[62,70,78]; however, Duncan and Douglas[38] found no effect. This suggests that if extracellular Ca^{2+} is involved in the development of basal tone it enters via a pathway other than VDCs (for example, via a "leak" pathway[109]). However, gallopamil in high concentrations (50 μM) substantially inhibited basal ^{45}Ca uptake in rabbit tracheal smooth muscle[10] whereas in guinea pig trachea, verapamil (1 μM) had no effect.[72]

Isolated Airway Smooth Muscle Cells in Suspension

Recently, contractile responses of suspensions of isolated airway smooth muscle and lung parenchymal cells have been examined in our laboratory. The cells were prepared by enzymatic digestion[110] of canine trachealis and guinea pig lung parenchyma. Contractile responses were measured using particle size analysis[111] to indicate changes in cell size. It was observed that

KCl and methacholine induced contractile responses. Verapamil (1 μM) antagonized the contractions, and to a degree similar to that seen in intact tissue preparations. In isolated cell suspensions verapamil reduced the KCl-induced maximum contraction to 55 ± 5% and 75 ± 4% of control in trachea and lung parenchyma, respectively.

Human Isolated Tissue Studies

Few studies on the role of Ca^{2+} and the effects of CEBs on human airway smooth muscle in vitro have been published. A summary of the findings in relation to the tissues studied, the antagonists and their concentrations, etc. is given in Table 3.1. Both the antispasmogenic and the spasmolytic actions of the drugs have been examined, but usually not in the same report. Generally, CEBs are much more effective as spasmolytic than as antispasmogenic agents, a phenomenon also evident in in vitro animal studies. We will first consider the antispasmogenic actions of CEBs.

Antispasmogenic Effects of CEBs

Nifedipine has been shown to antagonize human airway smooth muscle contraction induced by histamine,[112,113] acetylcholine,[113] LTC_4, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), and KCl.[114] The degree of inhibition, ie, the potency of nifedipine, varied with each agonist. KCl- and histamine-induced responses were the most greatly inhibited, followed by those to LTC_4, LTD_4, $PGF_{2\alpha}$, and acetylcholine. Similar results were obtained with PY108068, a newer 1,4-dihydropyridine CEB: KCl-induced contractions were the most greatly affected, followed by those to histamine, methacholine, and LTD_4.[115]

The antagonism by CEBs is manifest in agonist concentration–response curves as a shift to the right (ie, a reduction in agonist potency) and/or a depression of the maximum contractile response. These effects are dependent on the CEB concentration, the particular agonist, and the tissue. Quantifying the effect of a CEB, either in terms of changes in the sensitivity to the agonist or in its maximum response, is often difficult.[116] For example, large increases in EC_{50} and reductions in maximum response for histamine and acetylcholine in the presence of nifedipine have been reported.[113] This would suggest that nifedipine is very potent. However, these results[113] are affected by the method used to calculate the dose ratios, even though the effect of nifedipine on the curves is quite clear. Nifedipine reduced the maximum responses to histamine and acetylcholine, and the dose ratios were calculated by extrapolation to obtain the EC_{50}s in the presence of nifedipine. The resultant dose ratios for expressing the potency of nifedipine were, therefore, probably overestimated (see Fig. 2 in ref. 113). Thus, the use of EC_{50}s does not adequately describe the antagonism by nifedipine per se.

The antispasmogenic action of verapamil in human tissue appears to be similar to that of the dihydropyridine CEBs. Contractions of human bronchus induced by KCl and histamine[62] were the most easily antagonized by verapamil, whereas responses to LTC_4 and methacholine[115,117] were relatively unaffected. Ito et al[118] examined the antispasmogenic effects of verapamil on contractions induced by Ca^{2+} in tissues depolarized with high and low concentrations of KCl. In each case verapamil did not consistently affect the responses and, when it did, the effect was relatively slight.

Removal of the airway epithelium from human trachea reduced the antispasmogenic effect of verapamil,[105] thereby extending the finding from studies in rabbit airways.[33]

Spasmolytic Effects of CEBs

As described above, there are a number of reasons why antispasmogenic and spasmolytic effects of CEBs would be expected to differ. In support of this idea are findings demonstrating that, for a given concentration of CEB, the percentage of inhibition of response of a contracted tissue is greater than the reduction in maximum response seen when the antagonist is added before evoking the response. Nifedipine was shown to reverse completely the response of tracheal muscle to histamine[112,113] and of bronchial muscle to KCl.[113] Responses to acetylcholine[113] and LTC_4[114] were reduced by approximately 60%, whereas responses induced by LTD_4 and $PGF_{2\alpha}$[114] were reduced by less than 50%. These effects were greater than the antispasmogenic effects in the same tissue (Table 3.1). Gallopamil was shown[119] to inhibit contractions induced by LTC_4 and LTD_4 by approximately 50%. Studies in our laboratory have shown that the response to methacholine in tracheal muscle is inhibited substantially (60%) by verapamil (unpublished observations).

Of use to understanding the role of CEBs in asthma are experiments involving passively sensitized in vitro preparations of human airway smooth muscle. Using this technique it is possible to compare the actions of CEBs on the contraction of smooth muscle following an antigen–antibody interaction, mast cell degranulation, and mediator release, with that following the administration of exogenous drugs. Nifedipine produced a greater than 60% inhibition of contractions induced by acetylcholine and histamine and an approximately 50% inhibition of the response induced by a grass pollen antigen.[120] However, the concentration of nifedipine used (100 μM) in this study was very high and is probably not achievable in humans in vivo without untoward cardiovascular side effects.

The spasmolytic action of PY108068 on contraction induced by an antigenic extract of the antigenic house dust mite (*Dermatophagoides pteronyssimus*) has been examined in our laboratory (Fig. 3.2). PY108068 inhibited the response to *D. pteronyssimus* by approximately 20%.

TABLE 3.1 Summary of the Effects of CEBs on Responses of Human Airway Smooth Muscle in Vitro

Tissue	Status	Applied Load (g)	CEB (Concentration)	Agonist	% Inhibition of Maximum Response[a]	Dose Ratio	Δ Baseline Tension	Reference
Trachea, bronchus	Autopsy, surgery	L_o[b]	Gallopamil (5–10 μM)	LTC_4 LTD_4	40–50(s)	—[f]	—[f]	119
Trachea	Autopsy	8	Nifedipine (2.9 μM)	Histamine	50(a) 100(s)	10	—	112
Bronchus	Surgery	0.5[c]	Nifedipine (1–100 μM)	Acetylcholine Histamine Grass pollen	30–62(a) 53–77(a) 39–49(a)	—	No effect	120
Bronchus	Surgery	2	Nifedipine (2.9 nM–2.9 μM)	Histamine	58(a) 100(s)	160[g]	—	113
				Acetylcholine	28(a) 63(s)	22[g]		
Bronchus	Surgery	2	Verapamil (1 μM)	KCl Histamine LTC_4	55(a) 40(a) 22(a)	— 3 —	No effect	62
Bronchus	Surgery	2	Nifedipine (2.9 nM–2.9 μM)	LTC_4	47(a) 60(s)	—	—	114
				LTD_4	34(a) 40(s)			
				$PGF_{2\alpha}$	37(a) 30(s)			

				KCl	60(a) 100(s)			
Trachea	Autopsy	0.5[d]	Verapamil (30 μM)	Ca^{2+} (5.9 mM KCl) Ca^{2+} (40 mM KCl)	<60(a)[e] <70(a)[e]	—	—	118
Bronchus	Surgery	2	Verapamil (1 μM)	LTD_4 Methacholine	15(a) 30(a)	—	No effect	117
Bronchus	Surgery	2	PY108068 (0.01–1.0 μM)	KCl Histamine Methacholine LTD_4	35–63(a) 17–54(a) 17–31(a) 20(a)	—	No effect	115
Trachea	Autopsy	2	Verapamil (1 μM)	Methacholine	25	—	No effect	105

[a](s), spasmolytic effect; (a), antispasmogenic effect.
[b]L_o, experimentally determined optimal resting load.
[c]Responses measured isotonically (all others measured isometrically).
[d]Load applied in the presence of isoproterenol (2 μM) and tissues perfused.
[e]Responses very variable. To arrive at the data in this column, changes in tension of control tissues were taken into account.
[f]Not recorded.
[g]See text for further discussion.

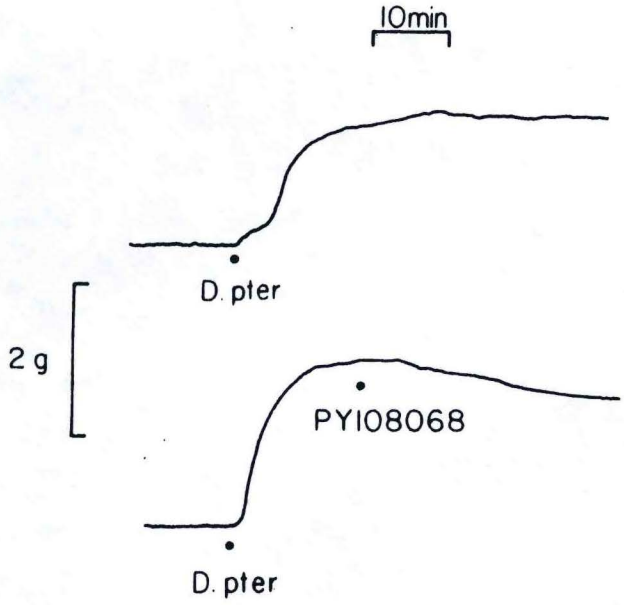

FIGURE 3.2 Spasmolytic effect of the 1,4-dihydropyridine CEB PY108068 on the contraction of human isolated bronchial smooth muscle induced by challenge with an antigenic extract of *D. pteronyssimus* (added where indicated by "D. pter"). The tissues had been passively sensitized with a high IgE titer serum obtained from an atopic donor. PY108068 (1 μM) caused a relaxation of the tissue (bottom panel), which was not evident in the vehicle-treated control (top panel).

In summary, CEBs are more effective as spasmolytics than as antispasmogenic agents.

Effects of La^{3+} and Removal of Extracellular Ca^{2+}

Although of no clinical value, La^{3+} is a useful tool for examining transmembrane Ca^{2+} movements. La^{3+} caused an antispasmogenic-type reduction in responses to LTD_4, methacholine, and histamine, and abolished contractions to KCl and the Ca^{2+} ionophore A23187.[115] Since La^{3+} blocks all Ca^{2+} entry into cells, these findings suggest that receptor activation may mobilize Ca^{2+} from a store within the cell. La^{3+} had no spasmolytic effects on responses to any of the agents.[115]

A reduction in the extracellular Ca^{2+} concentration produces substantial loss of tone in human airway smooth muscle[115]; however, CEBs do not have a similar effect on resting tone.[62,105,115,117,120] Thus, although the maintenance of resting tone is dependent on extracellular Ca^{2+}, the fact that CEBs have no effect suggests that Ca^{2+} influx through VDCs is not the mechanism involved. This situation is similar to what is seen in experimental animals. The effect of CEBs on baseline pulmonary function in vivo is discussed later.

In Vivo Studies

Animal Studies

Suprisingly few in vivo studies have been performed on the effects of CEBs on pulmonary function in experimental animals. From the data available it can be seen that CEBs have a wide range of activity, depending on the bronchoconstrictor agent used, its dose, the dose of CEB used, and the route of administration. As can be seen in Table 3.2, CEBs generally inhibit bronchoconstriction induced by receptor agonists (serotonin, histamine, $PGF_{2\alpha}$, muscarinic agonists, and LTD_4), by the nonspecific irritant citric acid, and by the antigenic substances *Ascaris suum* extract and ovalbumin in sensitized animals. The effects of CEBs administered by inhalation were similar to those seen when the drugs were given intravenously. In some cases the drugs' actions were more pronounced in certain areas of the respiratory tract. For example, intravenously administered nifedipine or verapamil inhibited *A. suum*-induced spasm in large but not in small airways[125]; aerosolized verapamil inhibited these responses in large and small airways, whereas nifedipine had no effect. In contrast, Russi et al[129] reported that verapamil had no effect on the bronchospasm induced by histamine or carbachol, yet it abolished the response to antigen. Similarly, Brugman et al showed attenuation of *A. suum*-induced responses by inhaled nifedipine.[126] Rounding and Towart[132] described effects of nifedipine that differed depending on the route of administration. Intravenous nifedipine was active against histamine-induced bronchospasm, whereas inhaled nifedipine had no effect. The discrepancies in these studies may be due to differences in methodology.

Human Studies

Human studies have focused mainly on the effects on CEBs in preventing exercise-induced asthma (EIA) and bronchoconstriction induced experimentally by various agents in asthmatic and control subjects, and on basal pulmonary function.

Exercise-Induced Asthma. The mechanisms involved in EIA have been reviewed recently.[136,137] In up to 90% of asthmatic patients, an acute attack will follow several minutes after beginning vigorous exercise. In addition, EIA may be precipitated in some overtly asymptomatic asthmatic individuals, especially those have chronic rhinitis. In most cases the bronchospasm reverses spontaneously within 60 minutes.

There is considerable debate about the pathogenesis of EIA. Inhaling cold, dry air during exercise can exacerbate the condition, whereas moist, warm air has a preventative effect. The current view[136,137] is that respiratory water loss and possibly heat loss act as a stimulus for bronchospasm. Water

TABLE 3.2 Summary of the in Vivo Effects of CEBs in Experimental Animals

Species	Provocation	CEB (Dose)	Route	Effect	Reference
Dog	•	Nifedipine (0.3–10 μg/kg)	IA	–	107
		Verapamil (0.3–10 μg/kg)	IA		
Cat	Serotonin	Diltiazem (0.4 mg/kg)	IV	–	121
Guinea pig	Histamine	Nifedipine (30 μg/kg)	IV	+	108
Dog	$PGF_{2\alpha}$ and histamine	Nifedipine (0.2–0.4 mg/kg)	IV	+	122
		(0.1–1.0%)	Inhalation		
Sheep	*Ascaris suum*	Verapamil (150 μg/kg)	IV	+	123
Dog	Citric acid and methacholine	Nifedipine (1.25 mg/ml)	Inhalation	+	124
Dog	*A. suum*	Nifedipine or verapamil (0.2 mg/kg)	IV	±	125
		(1.0%)	Inhalation		
Dog	Citric acid, methacholine, and *A. suum*	Nifedipine (1.25 mg/ml)	Inhalation	+	126
Guinea pig	Acetylcholine and LTD_4	Nicardipine or verapamil (0.3–1.0 mg/kg)	IV	–	127
Guinea pig	Histamine and acetylcholine	Nicardipine (0.3–1.0 mg/kg)	IV	±	128
		Verapmil (1.0 mg/kg)	IV	–	
Sheep	*A. suum*, histamine, carbachol	Verapamil (150 μg/kg)	IV	±	129
Guinea pig	Ovalbumin	Verapamil (4.0–8.0 mg/kg)	IV or IP	±	130
		Diltiazem (5.0–10.0 mg/kg)			
Guinea pig	Ovalbumin	Nifedipine (0.5 mg/kg)	IV	+	131
Guinea pig	Histamine and citric acid	Nifedipine (0.5 mg/kg)	IV	+	132
		(1.0–10.0 mg/kg)	Inhalation	–	
Dog	Histamine	Verapamil (10–100 μg)	Inhalation	+	133
Guinea pig	Ovalbumin and LTC_4	Nicardipine (7.0 μg/kg)	IV	±	134
Guinea pig	Ovalbumin and LTC_4	Nifedipine (7.0 μg/kg)	IV	+	135

•Basal pulmonary function measured.

Abbreviations: IA, intraarterial; IV, intravenous; IP. intraperitoneal; +, protective effect against bronchospasm; –, little or no protective effect; ±, effect depends on route of administration or provocation used.

loss leads to hyperosmolarity of the airway epithelium, and is probably more provocative for bronchoconstriction than airway cooling.

To elicit EIA, subjects are exercised or made to breathe cold air. The effects of CEBs are evaluated by examining their ability to interfere with the resulting bronchospasm.

Nifedipine, in doses and via routes effective in the treatment of cardiovascular diseases, has been shown consistently to afford protection against exercise- or cold-air-induced bronchoconstriction.[138-145] In no study, however, did nifedipine affect resting pulmonary function. This result is comparable to the lack of effect of dihydropyridine CEBs on basal tone in in vitro human airway preparations.[115,120] These reports suggest a potential role for nifedipine in the prophylaxis of EIA. However, the effectiveness of nifedipine depended on the intensity of the cold air challenge, and at high intensities nifedipine was not effective.[145]

Verapamil, like nifedipine, appears to exhibit some protective effect against EIA.[141,146-148] As was the case with nifedipine, however, verapamil did not produce a significant beneficial effect against intense thermal challenge.[145] Verapamil also was without effect on prechallenge pulmonary function. Other studies using diltiazem,[149] gallopamil,[148] and PY108068[150] have been performed. Diltiazem had no protective effect, whereas gallopamil showed a profile of action similar to verapamil in that it was effective in EIA. PY108068, albeit in very high doses, also was effective. There is clearly some beneficial effect to be obtained from CEBs in the control of EIA.

The mechanism of the protective effect of CEBs in EIA is unclear, as is the etiology of the symptoms. Possible actions of CEBs in EIA include an interference in excitation–contraction coupling in airway smooth muscle itself or the prevention of mediator release. A great deal of work is needed in this area.

Drug-Induced Bronchoconstriction. Studies of drug-induced bronchoconstriction have involved the inhalation administration of bronchoactive drugs such as histamine, methacholine, acetylcholine, carbachol, leukotrienes, and antigen to induce bronchospasm before and after the administration of a CEB. To date only the antispasmogenic actions of the CEBs have been evaluated.

Nifedipine has variable effects on histamine-induced bronchospasm. In most studies nifedipine attenuated responses to histamine to some degree.[139,143,151-153] On the other hand, some studies[141,154] were unable to demonstrate an effect of nifedipine on histamine-induced bronchoconstriction. A few studies using other CEBs have been performed. Nicardipine, a new dihydropyridine CEB, was shown to be without protective effect against

carbachol-induced bronchospasm.[155] On the other hand, diltiazem had some protective action against histamine and carbachol challenge in control and asthmatic subjects.[156]

As with histamine, responses to methacholine and acetylcholine are variably affected by nifedipine. The effects of this CEB ranged from no effect[157] to varying degrees of inhibition.[152,158-160] Interestingly, methacholine[161] induced a bronchoconstriction that was unaffected by verapamil, whereas the CEB blocked the spasm induced by acetylcholine.[162]

Histamine-induced responses were variably affected by the administration of verapamil. No effect[161] or a significant protective effect[162,163] have been reported. The time of administration of the CEB was shown to determine the degree of protective effect.[164] Verapamil given before histamine challenge afforded some protection, but not verapamil given during challenge. With the exception of Popa et al[162] who also examined nonasthmatic individuals, the above studies were conducted using asthmatic subjects.

Antigen-induced bronchospasm in susceptible individuals is variably affected by nifedipine. So et al[165] observed no protective effect against challenge with *D. pteronyssimus*. Responses to challenge with ragweed were partially inhibited,[166] whereas responses to grass pollen were significantly reduced.[120] Verapamil, on the other hand, had little or no effect on the response to inhaled antigen.[165,166]

Of interest are studies by Roberts et al[117,167] in which verapamil was shown to inhibit significantly the contraction induced by LTD_4 in normal subjects but had no effect in asthmatic subjects. This interesting observation clearly merits further study as it highlights the notion that fundamental physiological alterations occur in the airways in asthma.

Nonspecific Actions of CEBs

High concentrations of CEBs have been shown to produce a wide range of effects other than antagonism of Ca^{2+} entry through VDCs. These include interference with mediator release from mast cells; interactions with α-adrenergic, β-adrenergic, cholinergic, and serotonin receptors[168-171]; stimulation of Na^+,K^+-ATPase[172]; inhibition of transmitter release[173,174]; and local anesthetic activity.[175] In view of the high concentrations which are frequently used in experiments studying airway smooth muscle function both in vitro and in vivo, the nonspecific actions of the CEBs unrelated to their binding to VDCs could confound the interpretation of results. Some of these nonspecific actions are now discussed in more detail.

Inhibition of Mediator Release

Bronchoactive mediators released from mast cells, for example, the leukotrienes and histamine, are thought to play an integral role in the etiology of bronchial asthma (see, for example, ref. 176). The release process, as well as mediator synthesis, is Ca^{2+}-dependent, involving the transmembrane influx of Ca^{2+}. Mediator release can be activated by immunologic and nonimmunologic stimulation.[177-180] For example, antigenic challenge of mast cells was associated with increased ^{45}Ca uptake, and the ensuing mediator release could be decreased by reducing the extracellular Ca^{2+} concentration.[177-181]

There are conflicting reports on the ability of CEBs to inhibit mediator release from mast cells and other cells. Results ranging from no effect to total inhibition of release have been reported (see Table 1 in ref. 179). Verapamil, nifedipine, and gallopamil had no effect on histamine release from human basophils,[182] from passively sensitized lung tissue,[183,184] and from ovalbumin-sensitized guinea pig trachea.[185] In contrast, these compounds as well as nimodipine and nicardipine produced marked inhibition or abolition of mediator release, and the associated increase in ^{45}Ca uptake, from human basophils both in vivo and in vitro,[186-188] human lung tissue,[184-189] human neutrophils,[190] passively sensitized rhesus monkey lung,[42] and rat peritoneal mast cells.[191-193] However, in these studies the concentrations of the CEBs required to decrease mediator release were generally much higher than those required to inhibit smooth muscle contraction by a direct effect. Assuming that in both situations these compounds act by inhibiting Ca^{2+} entry, then this result suggests that mast cell and basophil Ca^{2+} channels differ from those in airway smooth muscle. It is therefore unlikely that the effects of CEBs on pulmonary function in vivo result in large part from an influence on mediator release, except when the drugs are used in high doses.

It was suggested that verapamil abolished the antigen-induced bronchoconstriction in *Ascaris*-sensitized sheep by preventing mast cell degranulation.[166] This hypothesis was based on the lack of effect of verapamil on histamine- and carbachol-induced bronchospasm, but it is equally likely that the bronchoconstriction produced by antigen challenge involves other mediators in addition to histamine, for example, the leukotrienes. Nifedipine inhibited the elevation of plasma histamine observed in EIA,[139] suggesting an effect on mediator release, but the source of the released histamine is unknown. It is of interest that verapamil and nifedipine in reasonably low concentrations (5–10 μM) decreased the IgE-mediated release of leukotrienes from passively sensitized human lung but had no effect on histamine release.[183,189] It is not known if this difference is related to the fact that the leukotrienes are synthesized and released de novo, whereas previously synthesized histamine is stored in granules, or if it reflects differences in the Ca^{2+}

channels involved in their release. Verapamil, but not nifedipine, has been reported to inhibit 5-lipoxygenase activity,[130] and an effect on Ca^{2+}-dependent leukotriene synthesis cannot be ruled out.[43]

Inhibition of Phosphodiesterase

Verapamil, nifedipine, and diltiazem have been shown to be taken up by smooth muscle, which suggests that the drugs might have intracellular actions.[194]

Evidence has been obtained suggesting that cyclic AMP phosphodiesterase activity is inhibited by certain CEBs. By elevating intracellular cyclic AMP concentration, such an effect would promote bronchodilation. Dihydropyridines in relatively low concentrations (nifedipine $IC_{50} = 2\ \mu M$) inhibited the activity of calmodulin-sensitive (peak I) cyclic AMP phosphodiesterase from bovine hearts; this effect was selective in that concentrations $\geq 100\ \mu M$ were required to inhibit calmodulin-insensitive cyclic AMP phosphodiesterase (peak II) activity of the enzyme. Verapamil, gallopamil, and diltiazem were ineffective against both forms of the enzyme ($IC_{50} > 100\ \mu M$).[195] In contrast, it was reported that nimodipine and nicardipine in micromolar concentrations inhibited competitively both the calmodulin-sensitive and calmodulin-insensitive forms of cyclic AMP phosphodiesterase; verapamil was nearly equipotent in inhibiting peak II phosphodiesterase activity, but was much less effective as an inhibitor of peak I activity.[196] The differences between the two studies[195,196] may be related to the different sources of the enzymes. To further complicate the issue, another study revealed that verapamil, nifedipine, and diltiazem in clinically relevant concentrations had no effect on phosphodiesterase activity from bovine heart, both in the presence and the absence of calmodulin.[197]

In experiments using guinea pig trachea in vitro, nifedipine ($10\ \mu M$) increased the sensitivity of tissues to the relaxant effects of isoproterenol.[198] In other studies on this tissue, however, nifedipine and isoproterenol antagonized each others' relaxant effects.[199] Furthermore, nifedipine ($0.1\ \mu M$) abolished the isoproterenol-stimulated increase in the levels of cyclic AMP.[200] Duncan and Douglas[38] found no effect of nitrendipine on the spasmolytic effects of isoproterenol and aminophylline in guinea pig airways in vitro. An interesting finding of the study by Daya and Joubert[199] was that, unlike nifedipine, the relaxant effects of verapamil on guinea pig trachea contracted by histamine or methacholine were potentiated by isoproterenol and theophylline.

This in vitro interaction between CEBs and β-adrenoceptor agonists introduces the possibility of achieving a greater bronchodilatory effect in vivo by administering a combination of a β-adrenoreceptor agonist and a CEB, both at lesser doses to reduce side effects, than is produced by either

agent alone. It is worth noting that nifedipine prolongs the duration of bronchodilation by salbutamol in asthmatics,[201] and increases the sensitivity of subjects to intravenous terbutaline.[202] This action, rather than one involving a direct dilation of the airways, may be of some benefit in the management of asthma.

Inhibition of Neurotransmitter Release and Actions

Although high concentrations of CEBs can reduce transmitter release[173,174] nifedipine and felodipine (both 10 μM) did not affect the basal, KCl-, or Ca^{2+}-stimulated release of [^{3}H]norepinephrine from rat trachea[203]; however, felodipine (100 μM) inhibited stimulated release. These findings are indicative of the relative insensitivity of neuronal Ca^{2+} channels to CEBs compared with those of the smooth muscles. Verapamil and gallopamil, but not nifedipine, possess local anesthetic activity.[175] It is possible that by this action verapamil may inhibit airway neurotransmitter release, and in vivo may ameliorate bronchospasm produced by the vagal reflex.

Nifedipine produces an atropine-sensitive contraction of guinea pig trachea in the presence of isoproterenol.[199] Similarly, it was reported that high doses of verapamil produced in vivo bronchoconstriction in sheep; this response was blocked by atropine.[129,204] Furthermore, high doses of verapamil produced appreciable bronchoconstriction in humans.[205,206] These observations suggest that under certain conditions CEBs may, in fact, promote neurotransmitter release.[207]

Muscarinic Receptor Antagonism

Verapamil, but not nifedipine, produced a dose-dependent inhibition of 3-quinuclidinyl benzylate ([^{3}H]QNB) binding to muscarinic receptors in bovine tracheal smooth muscle. It was suggested that this effect may contribute to the antagonistic effects of high concentrations of verapamil on responses following muscarinic receptor activation in airway smooth muscle.[208,209] Obviously, if verapamil is used to identify sources of Ca^{2+} for muscarinic receptor-induced contractions, concentrations of verapamil must be used that do not cause muscarinic receptor blockade.

Pharmacokinetic and Therapeutic Considerations

In view of the high concentrations of the CEBs generally required to alleviate airway smooth muscle contraction in vitro and in vivo, the beneficial effect of these drugs in asthma and other respiratory diseases may be offset by their untoward cardiovascular actions. Most human and animal in vivo studies of nifedipine have used the oral or sublingual administration of doses (10 or 20 mg) used in the management of certain cardiovascular disorders; therefore,

cardiovascular side effects have been associated with the use of CEBs for treating respiratory diseases.[120,142,155] However, cardiovascular side effects after the oral administration of nifedipine (20 mg) were not noted.[151]

The beneficial effects against bronchoconstriction evoked by a variety of stimuli of orally administered nifedipine are generally small and are less than the protection afforded by conventional therapy with β-adrenoceptor agonists (see for example ref. 151). This suggests that orally administered nifedipine has limited value in the treatment of asthma.

Administration of CEBs by inhalation should offer several advantages over the oral or intravenous routes. Inhalation of the drugs provide a rapid and localized deposition of a high drug concentration at the target organ, and should result in lower plasma levels and fewer side effects. Furthermore, potential difficulties arising from inconsistent or incomplete absorption from the gastrointestinal tract should be circumvented. Unfortunately, studies with aerosolized nifedipine and verapamil have produced conflicting and disappointing results.[139,143,151,153,154] This lack of a consistent benefit from the inhaled drug suggests that the small effect of these drugs when administered orally or sublingually is not due to insufficient concentrations reaching the respiratory tract per se, but rather is intrinsic and is related to the minor role of Ca^{2+} influx through VDCs in the development of bronchospasm. An additional feature of inhaled verapamil, which may further preclude its use, is that it appears to be an irritant.[162] Overall, the inhalational administration of nifedipine and verapamil has not yielded the beneficial effect that was hoped for on the basis of pharmacokinetic considerations.

The cardiovascular side effects of CEBs may however, be advantageous in patients who suffer from both asthma and a cardiovascular disorder. Treatment of the respiratory distress with β-adrenoceptor agonists may worsen the cardiovascular symptoms, whereas β-adrenoceptor antagonists used in cardiovascular disorders may lead to bronchoconstriction (see ref. 206 and references inter alia). Treatment with CEBs may be of real benefit to patients who have both angina and mild to moderate symptoms of asthma.[158,210]

The possibility of increasing the therapeutic effectiveness of CEBs in asthma by using long-term treatment is now being evaluated. Nifedipine given for 3 weeks reduced the severity of asthmatic attacks but did not reduce their incidence; basal respiratory function was unaltered as was the case when the drug was given acutely.[211] Further evidence for a potential role of chronic treatment with CEBs was provided by the recent observation that asthmatic symptoms worsened after cessation of nifedipine therapy in two patients with angina pectoris and chronic obstructive lung diseases.[212] However, no greater benefit was seen with nifedipine given over an extended period than from acute administration.[142]

The possibility exists that the CEBs, especially if used chronically, may affect other Ca^{2+}-dependent processes in the respiratory tract besides smooth muscle contraction and mediator release, for example, mucus secretion, chemotaxis of inflammatory cells, and epithelial ion and water transport. This possibility has not been systematically analyzed, although verapamil was reported to have no effect on the antigen-induced decrease in tracheal mucus velocity in an ovine model of allergic asthma.[206]

The beneficial effects of long-term therapy with CEBs in respiratory tract disorders would appear to be an important area of investigation.

Possible Differences Between the Ca^{2+} Channels of Airways and of Other Smooth Muscles

K^+-induced contractions of airways, which depend on extracellular Ca^{2+}, are less sensitive to CEBs than those of several vascular and visceral smooth muscles.[213,214] This fact suggests that there may be differences in the voltage-dependent Ca^{2+} channels that render the airways more resistant to the effects of CEBs than other types of smooth muscles. The effect does not appear to be due to differences in the affinity of the CEBs for the Ca^{2+} channels, as [^{3}H]nitrendipine binds to guinea pig lung[215] and bovine tracheal smooth muscle membranes[209] with affinities (K_D = 0.18 nM and 0.15 nM, respectively) similar to those observed in other smooth muscles.[215] However, the number of [^{3}H]nitrendipine binding sites in airways is less than that observed in other smooth muscles.[209,215] Although there are no significant tissue differences in the affinity of the dihydropyridines for the Ca^{2+} channel in its resting state, it is possible that differences in the binding of CEBs arise after channel activation, for example, with elevated K^+ concentrations.[216] Thus, in airway smooth muscle there may be two main factors contributing to the relative ineffectiveness of CEBs in vitro and in vivo: (1) reliance of responses predominantly on an intracellular Ca^{2+} source, and (2) inherent low sensitivity of VDCs.

Future Directions

The Phosphatidylinositol Pathway in Airway Smooth Muscle

Evidence has accumulated recently indicating that stimulation of membrane-associated phosphatidylinositol turnover plays a pivotal role in a multitude of receptor-mediated events in numerous cell systems.[217–219] Increased phosphatidylinositol turnover may be involved in pharmacomechanical coupling in airway smooth muscle, perhaps linked to ROCs.[220] The phosphatidylinositol pathway involves the rapid formation of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), which are currently

thought to be important intracellular "second messengers."[217-219] IP_3 may mediate the release of intracellular Ca^{2+} and also play a role in the entry of extracellular Ca^{2+}.[217-219] Exogenous IP_3 evoked the release of Ca^{2+} from intracellular stores in saponin-permeabilized airway smooth muscle cells.[221] DAG, which activates protein kinase C and stimulates phosphorylation of specific proteins, may also be involved in controlling Ca^{2+} translocation mechanisms associated with the sustained phase of smooth muscle contraction.[217,219,222-224]

Carbachol-induced contraction of canine trachea is associated with increased phosphatidylinositol turnover.[220] In contrast, KCl-induced contractions were not accompanied by an increase in phosphatidylinositol turnover. This suggests that the phosphatidylinositol pathway is independent of changes in membrane potential, ie, that it is not involved in electromechanical coupling.[220]

Cholinergic agonists and 5-hydroxytryptamine were found to stimulate the formation of IP_3 and phosphatidic acid in dog airway smooth muscle, whereas histamine and $PGF_{2\alpha}$ did not.[221,225] The increase in IP_3 formation preceded rather than followed the onset of contraction, suggesting that IP_3 may have been involved in initiating contraction. This finding suggests that at least in dog airway smooth muscle, not all agonists that mobilize intracellular Ca^{2+} do so via an increased turnover of plasma membrane phospholipids.

Evidence was recently obtained from dog trachea suggesting that protein kinase C, a Ca^{2+}-dependent, calmodulin-independent enzyme,[226] may be involved in maintaining the secondary phase of contraction which is dependent upon extracellular Ca^{2+}.[223,224]

It appears that new agents that prevent the synthesis or action of IP_3 or interfere with the activation of protein kinase C, might be useful for preventing bronchoconstriction by interfering with these newly described mechanisms.

Intracellular Ca^{2+} "Antagonists"

Agents purported to act as intracellular Ca^{2+} antagonists inhibit contractile responses of airway and other smooth muscles, but they lack potency and specificity. For example, TMB-8 in high concentrations antagonized LTD_4- and arachidonic-acid-induced contractions of guinea pig trachea and lung parenchyma[40,43,63,100] and LTD_4-induced responses of rhesus monkey trachea.[42] However, TMB-8 also inhibited or abolished KCl-induced responses[40,42] and decreased LTD_4-stimulated thromboxane release. Unlike nifedipine and verapamil, TMB-8 administered intratracheally did not inhibit histamine- or antigen-induced bronchoconstriction in guinea pigs and rats.[227]

The calmodulin antagonist W-7 had no effect on contractions of guinea-pig trachea produced by leukotrienes.[76] Another calmodulin antagonist, trifluoperazine, inhibited agonist- and antigen-induced contractions of airways from ovalbumin-sensitized guinea pigs, but only in large concentrations ($\geq 100\,\mu M$).[44] New generations of compounds for modulating intracellular Ca^{2+} translocation pathways will need greater specificity and potency than the current ones if they are to be of use in respiratory tract disorders.

Concluding Remarks

Asthma is a complex, multifaceted, and multiphasic disorder involving numerous mediators and mechanisms; it exhibits profound interindividual and perhaps within-individual variability. This makes effective treatment using one strategy and a single class of compounds difficult.

The involvement of several mediators precludes the classical pharmacologic approach of using a single receptor antagonist to alleviate bronchospasm. It could necessitate rather the use of a battery of specific antagonist drugs, with the ensuing problems associated with combination therapy. An alternative approach is to intervene at a site between receptor stimulation and activation of the contractile machinery. The numerous liabilities of bronchodilator therapy with β-adrenoceptor agonists suggests that there is a need to develop new compounds specifically for use in respiratory disorders. CEBs were considered as possible candidates because the contractile tone of the airways depends on the level of cytosolic Ca^{2+}, and so it seemed that by reducing Ca^{2+} influx these agents would produce bronchodilation. However, even after inhalation, the presently available agents have been shown to possess limited and inconsistent clinical efficacy except in EIA, and are substantially less effective than β-adrenoceptor agonists. This ineffectiveness is probably primarily due to the fact that the mediators responsible for bronchoconstriction use mainly intracellular Ca^{2+} for contraction. In addition, the contribution of extracellular Ca^{2+} may be largely mediated by translocation via ROCs, which are inherently insensitive to CEBs. Furthermore, VDCs in airway smooth muscle may have different properties from those in other smooth muscle types.

New CEBs with selectivity for airways would be unlikely to produce substantially greater benefit than the presently available agents, and are not a promising long-term research goal. CEBs may be of significant clinical effectiveness in patients with both cardiovascular and mild to moderate obstructive lung disorders, where the use of β-adrenoceptor agents or antagonists is limited by possible adverse effects. There are several possible alternative strategies that might be useful for developing effective bronchodilator drugs, that are still based on the "Ca^{2+} hypothesis" of asthma, and that will share the

common property of reducing the cytostolic Ca^{2+} concentration or preventing the consequences of elevated intracellular Ca^{2+}. First, one might try to develop new and selective CEBs acting on ROCs. However, as is the case of drugs acting at VDCs, their effectiveness probably would be limited by the fact that several receptor-linked mediators are involved, each one capable of evoking intracellular Ca^{2+} release. Second, one might develop promoters of Ca^{2+} transport, Ca^{2+} efflux, and/or Ca^{2+} sequestration mechanisms; or third, inhibitors of intracellular Ca^{2+} release mechanisms, in particular inhibitors of steps in the phosphatidylinositol pathway. The second and third alternatives have the advantage that they are potentially able to prevent increases in cytostolic Ca^{2+} from intracellular stores in response to the plethora of candidate mediators implicated in asthma.

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Edited by

Mannfred A. Hollinger, Ph.D.
Professor
Department of Pharmacology
School of Medicine
University of California, Davis

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