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Activation of tracheal smooth muscle contraction: Synergism between Ca^{2+} and activators of protein kinase C

(phorbol esters)

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ABSTRACT **The effects of divalent ionophores (A23187 and ionomycin), Ca^{2+} channel agonist (BAY K 8644), and protein kinase C (C-kinase) activators [phorbol 12-myristate 13-acetate (PMA), mezerein] on bovine tracheal smooth muscle contraction were investigated. A23187 (5 μM) and ionomycin (0.5 μM) produced a prompt but transient contraction. C-kinase activators either produced no effect—e.g., PMA at 200 nM—or produced a rise in tension that was slow in onset but then gradually increased—e.g., mezerein at 400 nM. In contrast, ionophores and C-kinase activators, in combination, acted synergistically to produce a prompt and sustained contractile response that is reminiscent of that observed in response to carbachol, a cholinergic agonist. In addition, BAY K 8644 (20 nM), which has a minimal effect on tension by itself, could significantly enhance contraction induced by C-kinase activators. The contraction induced by all of these agents was quickly reversed either by removal of extracellular Ca^{2+} or upon addition of forskolin, an activator of adenylate cyclase. A similar reversal of carbachol-induced contraction by forskolin was observed with carbachol-induced contraction. These findings strongly suggest that C-kinase plays an important role in mediating tracheal smooth muscle contraction.**

Contraction of smooth muscle in response to appropriate agonists is thought to be mediated by an increase in the concentration of intracellular free Ca^{2+}. It has been proposed that the critically important Ca^{2+}-mediated step is at the level of the calmodulin-regulated protein kinase, myosin light chain (MLC) kinase (1, 2). Phosphorylation of the 20-kDa MLC by this enzyme is thought to initiate the interaction between actin and myosin in smooth muscle (1, 2). Since contraction of smooth muscle in response to agonist is sustained for the period of stimulation, this model implies that both the level of intracellular free Ca^{2+} and the amount of MLC phosphorylated should remain elevated for the duration of agonist action.

Recently, however, Morgan and Morgan have reported that addition of either phenylephrine or angiotensin II to isolated vascular smooth muscle strips leads to an immediate increase in intracellular free Ca^{2+} concentration, which peaks in 1–3 min and then falls to a value close to the original basal value (3, 4). However, contraction is sustained even when the intracellular Ca^{2+} concentration is close to its original basal value (3, 4). Likewise, the level of phosphorylated MLC in tracheal and vascular smooth muscle reaches a peak within minutes after agonist addition and then falls gradually to near basal values despite the sustained contractile response (5, 6). These results led to the postulate that a second calcium-dependent pathway, other than phosphorylation of MLC, operates during the sustained phase of smooth muscle contraction (5).

Results from recent work in a variety of secretory systems, including serotonin secretion from platelets (7), prolactin secretion from pituitary cells (8–10), insulin secretion from islet cells (11, 12), aldosterone secretion from adrenal cells (13–15), and amylase secretion from pancreatic acinar cells (16), have led to the development of a model of the calcium messenger system in which there are two branches by which information flows from cell surface to cell interior—(*i*) a calmodulin branch that is transiently activated by a transient rise in cytosolic free Ca^{2+} and is largely responsible for initiating cellular response and (*ii*) a protein kinase C (C-kinase) branch that is activated by the transient rise in Ca^{2+} and an increase in the diacylglycerol content of the plasma membrane and is largely responsible for sustaining the response (17, 18). Furthermore, a study of the regulation of the sustained phase of aldosterone secretion from adrenal glomerulosa cells suggests that the magnitude of this sustained response is determined by two factors: (*i*) the amount of activated, Ca^{2+}-sensitive form of C-kinase associated with the plasma membrane and (*ii*) the rate of Ca^{2+} cycling across this membrane (15).

One type of data used to develop this model of calcium messenger system function is that obtained from the use of agents that bypass receptor-mediated events in these tissues and directly activate either the calmodulin-dependent enzymes (e.g., A23187, ionomycin) or the C-kinase enzyme [e.g., phorbol 12-myristate 13-acetate (PMA), mezerein, 1-oleoyl-2-acetyl-glycerol] (6–12, 15, 19) or specifically increase the rate of Ca^{2+} influx by means of voltage-sensitive channels (e.g., a Ca^{2+} channel "agonist," BAY K 8644) (20). In several of the tissues or cells studied, stimulation with A23187 leads to a transient and submaximal secretory response and stimulation with PMA leads to a slowly developing and submaximal response (7–13, 16). In contrast, stimulation with the combination of A23187 and PMA leads to a response quantitatively and qualitatively similar to that induced by agonist (7–13, 16).

In previous work, an effect of PMA on contraction in vascular smooth muscle was reported (21, 22). The present study was undertaken to determine whether C-kinase activators (PMA or mezerein), alone or in combination with Ca^{2+} ionophores (A23187 or ionomycin), would induce contractile responses in bovine tracheal smooth muscle. Having found that these agents, in proper concentrations, induced contractile responses, an additional study was performed in which the effects of BAY K 8644 on PMA- and mezerein-induced contractions were determined. Our results strongly suggest that C-kinase is a second Ca^{2+}-sensitive pathway by which tracheal smooth muscle contraction is regulated.

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Abbreviations: MLC, myosin light chain; PMA, phorbol 12-myristate 13-acetate.

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MATERIALS AND METHODS

Fresh tracheae from 2- to 4-month-old calves were obtained from a local abbatoir and transported at 0°C in (a modified) Tyrode buffer with the following composition: 137 mM NaCl, 11.9 $NaHCO_3$, 5.5 mM dextrose, 0.3 mM NaH_2PO_4, 2.0 mM KCl, 2.25 mM $CaCl_2$, 1.0 mM MgCl (22, 23). Trachealis smooth muscle strips were prepared as described by Silver and Stull (6) by dissecting away the intimal and adventitial layers. All dissections were done just prior to use and no tissue older than 2 days was used. Tissue from 2-day-old tracheae responded normally to carbachol. Transverse strips (2- to 3-mm cross-sectional width × 6- to 7-mm length) were mounted vertically in a jacketed muscle bath (36 ± 1°C) containing Tyrode buffer aerated with 95% O_2/5% CO_2. A passive force of 1.5 g was applied to the strips and they were allowed to equilibrate for at least 2 hr. After this period, passive tension was readjusted to about 1.5 g and the strips were challenged with pharmacologic agents when passive tension became stable. When agents other than carbachol were used as stimulant, contraction studies were done in the presence of 0.1 μM atropine sulfate, which was added 5 min prior to addition of the stimulant. Tension was measured isometrically with Grass FT-03 force-displacement transducers or Statham strain gauges (model UC3) and was displayed on a Gould 2400S recorder with built-in preamplifiers. Since tissue from different sized tracheae exhibited slightly different contractility (as determined by differences in response to carbachol), all comparative studies were done by using strips obtained from the same trachea. Experiments in Ca^{2+}-free medium were done by using Tyrode buffer in which $CaCl_2$ was substituted with 1 mM EGTA.

All drugs were added to the organ bath (10 ml) in volumes not exceeding 10 μl. Atropine sulfate (Sigma), diphenhydramine hydrochloride (Sigma), and carbachol (Sigma) were dissolved in H_2O. PMA (Sigma), mezerein (LC series), and A23187 (Calbiochem-Behring) were dissolved in dimethyl sulfoxide. Ionomycin (Calbiochem-Behring) and forskolin (Calbiochem-Behring) were dissolved in ethanol. BAY K 8644 (Miles) was dissolved in polyethylene glycol to a concentration of 1 nM.

RESULTS

Effects of A23187 and PMA. As is shown in Fig. 1 *Upper Left*, when tracheal smooth muscle strips were exposed to 5 μM A23187, a prompt contractile response was observed, which peaked in ≈20 min and then gradually declined with time to a steady-state tension slightly above baseline by 60 min. In most cases, the active tension (i.e., measured tension − basal tension) after 75 min of ionophore stimulation was ≈30% of peak active tension (Table 1).

In contrast, no response was observable when muscle strips were exposed to 200 nM PMA for >2 hr (Fig. 1 *Upper Right*). Raising the dose of PMA to 500 nM, however, resulted in a very slow and progressive contraction that was observable only after 1–2 hr of drug exposure (results not shown).

The response of muscle strips to simultaneous exposure of A23187 (5 μM) and PMA (200 nM) was strikingly different from that observed in response to these two drugs individually. There was a prompt contractile response similar or greater in magnitude to that seen with A23187 alone, which was not readily reversed even after 2 hr (Fig. 1 *Lower Left*). In repeated trials, the active tension after 75 min of stimulation with A23187 and PMA was twice that observed in strips stimulated with A23187 alone (Table 1). Thus, there is an apparent synergism between A23187 and PMA in maintaining active tension of tracheal smooth muscle, and the resulting

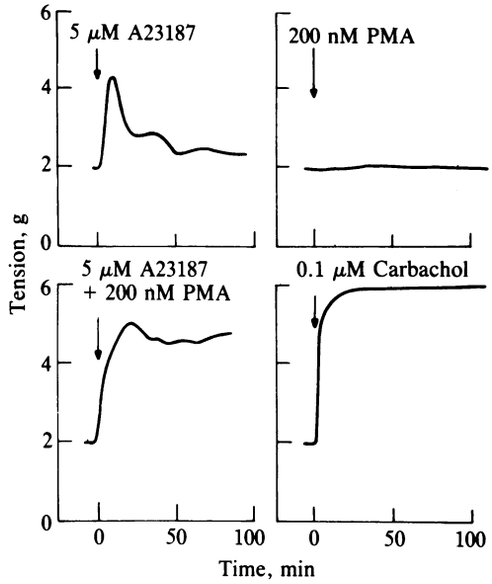

FIG. 1. Tracheal smooth muscle contractile response to A23187 and/or PMA. Fresh bovine tracheal smooth muscle strips were used for each experiment, and strips used here were dissected from the same trachea. All drugs were added at time = 0. In all groups except the carbachol group (*Lower Right*), 0.1 μM atropine was added 5 min before drug addition.

response is reminiscent of that seen with 0.1 μM carbachol stimulation (compare Fig. 1 *Lower Left* and *Lower Right*).

Effects of Ionomycin and Mezerein. Results similar to that obtained with A23187 and PMA were seen when a different calcium ionophore and C-kinase activator were employed (Fig. 2). Addition of 0.5 μM ionomycin led to a prompt rise in tension that peaked in ≈20 min and gradually declined to a plateau after 60 min (Fig. 2 *Upper Left*). When responses to ionomycin in different strips were averaged, the active tension after 75 and 120 min of exposure to the ionophore was 30% and 34% of the peak active tension, respectively (Table 1). Addition of 400 nM mezerein alone led to a slowly developing, progressive, and sustained response that was observable after a 10- to 20-min latency (Fig. 2 *Upper Right*). In contrast, combined addition of 0.5 mM ionomycin and 400 nM mezerein led to an initial sharp rise in tension that was followed by a slight fall and then a progressive and sustained response (Fig. 2 *Lower Left*). In repeated trials, the magnitude of response seen after the combined addition of ionomycin and mezerein was greater, at any point in time, than the sum of the responses to the individual stimulant (Fig. 2, Table 1). A similar synergism was seen when A23187 and mezerein were used in combination (results not shown).

Effects of BAY K 8644 and Mezerein. When the calcium channel agonist BAY K 8644 was employed with mezerein in place of divalent ionophores, a synergist response was also seen. The data in Fig. 3 show that the addition of 400 nM mezerein caused a slowly developing response (Fig. 3 *Upper Left*) and that 20 nM BAY K 8644 led to a slight early response that reached a plateau with time (Fig. 3 *Upper Right*). In contrast, addition of 20 nM BAY K 8644 and 400 nM mezerein simultaneously led to a more rapidly developing contraction and greater response than that seen with mezerein alone (Fig. 3 *Lower Left*). The active tension after 60 and 120 min in response to combined addition of BAY K 8644 and mezerein was greater than the sum of the responses resulting from each of the agents acting independently at these time points (Table 1). Interestingly, the response pattern was different if BAY K 8644 was added to strips that had been preincubated with mezerein for 2 hr. Addition of 20 mM BAY K 8644 led to a prompt increase in tension followed

Table 1. Summary of results obtained with various drug combinations

Drug	n	Active tension, g		
	Ionophore/C-kinase activator			
		At peak	At 75 min	At 120 min
A23187, 5 μM	4	3.4 ± 0.5	1.2 ± 0.5	—
PMA, 0.2 μM	4	0 ± 0	0 ± 0	—
+ A23187, 5 μM	4	3.7 ± 0.4	2.4 ± 0.2	—
Ionomycin, 0.5 μM	5	5.9 ± 1.0	1.8 ± 0.4	2.0 ± 0.6
Mezerein, 0.4 μM	3	—	3.2 ± 1.2	4.2 ± 1.0
+ Ionomycin, 0.5 μM	3	8.7 ± 0.4	9.2 ± 0.5	9.7 ± 0.7
	Ca^{2+} channel agonist/C-kinase activator			
		At 10 min	At 60 min	At 120 min
BAY K 8644, 20 mM	3	0.3 ± 0.1	0.8 ± 0	0.9 ± 0.2
Mezerein, 0.4 μM	3	0 ± 0	0.4 ± 0.1	0.9 ± 0.2
+ BAY K 8644, 20 nM (simultaneous addition)	3	0.2 ± 0.2	1.9 ± 0.5	3.5 ± 1.1
+ BAY K 8644, 20 nM (sequential addition)	5	3.8 ± 1.0*	—	—

Experiments were run as described in the legends to Figs. 1–3. Reported active tension refers to the difference between measured tension and basal tension at different periods following drug addition. The "peak tension" with ionophore stimulation refers to the maximal tension developed during the early, prompt contractile response that occurred within 20 min of drug addition. Values are reported as mean ± SEM.

*Value represents the change in tension noted 10 min following BAY K 8644 addition in tissues pretreated with mezerein.

by a slower progressive increase (Fig. 3 *Lower Right*). Similar synergism was noted with 20 nM BAY K 8644 and 500 nM PMA (results not shown).

Effects of Forskolin on Drug-Induced Responses. To demonstrate that the contractile responses elicited with ionophores, Ca^{2+} channel agonist, and C-kinase activators were physiologically significant, the reversibility of these responses with forskolin, an adenylate cyclase activator, was studied. Addition of 25 μM forskolin could completely reverse contraction resulting from the combined addition of ionomycin and mezerein (Fig. 4 *Upper*) or from sequential addition of mezerein and BAY K 8644 (Fig. 4 *Lower*). The forskolin-induced relaxation in these tissues was very rapid—response complete within 5 min—and was similar in magnitude and time course to the action of forskolin on carbachol-contracted tissues (results not shown).

Effect of Removing Extracellular Ca^{2+} on Contraction Induced by Mezerein and BAY K 8644. The contractile response to carbachol was diminished and transient in the absence of extracellular Ca^{2+}, suggesting the importance of extracellular Ca^{2+} in maintaining the agonist-induced contractile response (data not shown). In the case of contraction induced by mezerein and BAY K 8644, a similar Ca^{2+} dependency was seen. The data reproduced in Fig. 5 *Top* show that contraction induced by sequential stimulation of the muscle strips with mezerein and BAY K 8644 could be completely reversed within 20 min after removing extracellular Ca^{2+}. Subsequent readdition of extracellular Ca^{2+} resulted in a prompt and complete return of muscle tension.

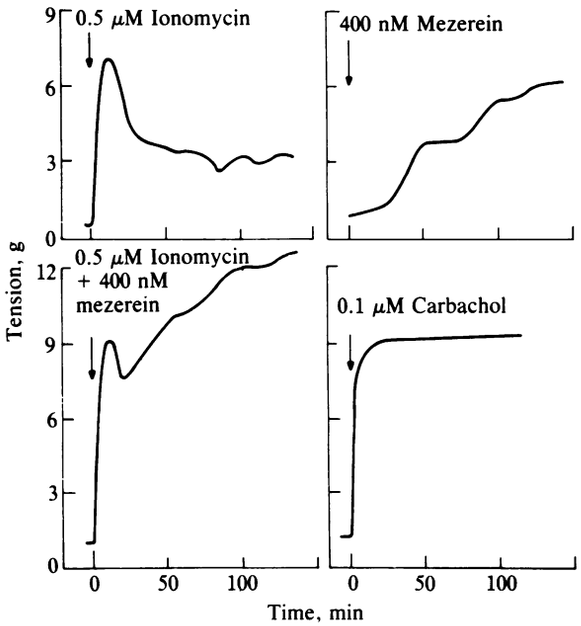

FIG. 2. Tracheal smooth muscle contractile response to ionomycin and/or mezerein. Conditions were as in Fig. 1. All drugs were added at time = 0 and 0.1 μM atropine was added 5 min prior to drug addition, as in Fig. 1.

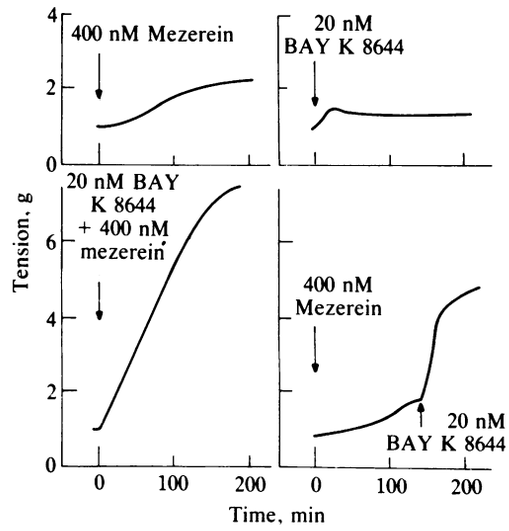

FIG. 3. Tracheal smooth muscle contractile response to BAY K 8644 and/or mezerein. Conditions were as in Fig. 1. All tissues were exposed to 0.1 μM atropine 5 min prior to drug addition. All drugs were added at time = 0 except for the group in which BAY K 8644 was added 2 hr after mezerein addition, as indicated (*Lower Right*).

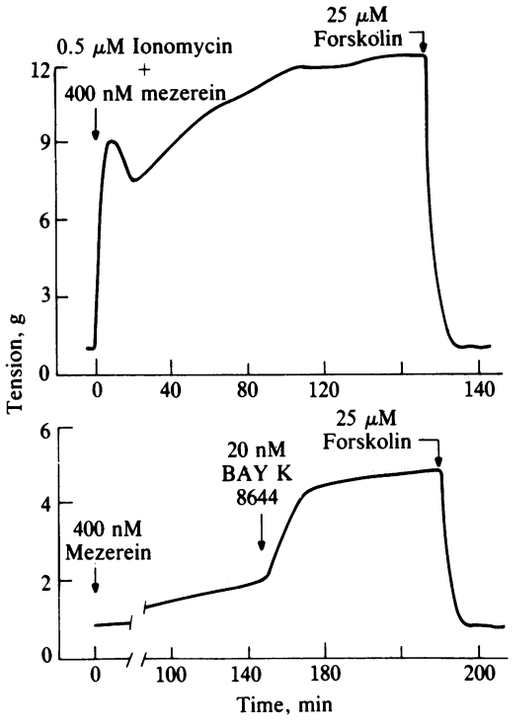

FIG. 4. Reversal of drug-induced tracheal smooth muscle contraction by forskolin. Forskolin was added at indicated times. (*Upper*) Condition as in Fig. 2 *Lower Left*. (*Lower*) Condition as in Fig. 3 *Lower Right*.

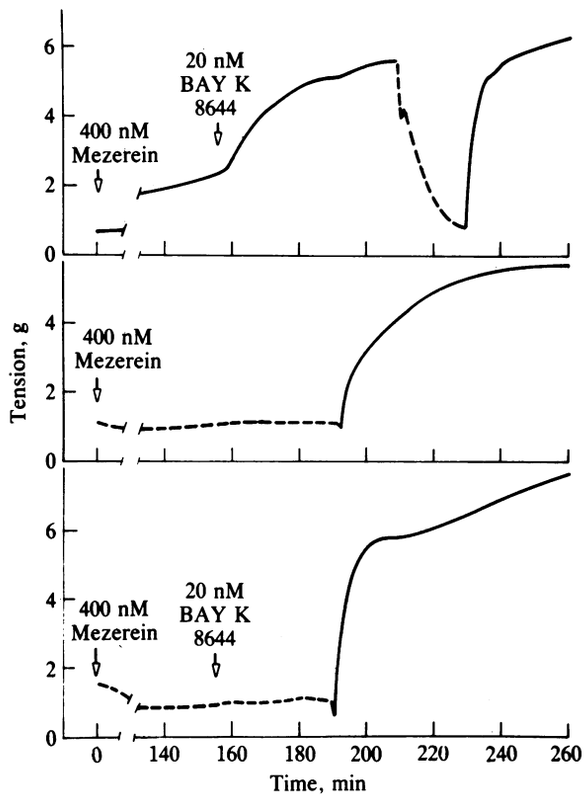

FIG. 5. Inhibition or reversal of drug-induced contraction by removal of extracellular Ca^{2+}. In each experiment 400 nM mezerein was added at time = 0, and 20 nM BAY K 8644 was added 155 min later to strips shown in *Top* and *Bottom*. (*Top*) Strips were incubated in the standard Ca^{2+}-containing buffer and responded to combined mezerein and BAY K 8644. At 208 min, the incubation medium was switched to a Ca^{2+}-free, 1 mM EGTA medium, and at 230 min, the Ca^{2+}-free medium was replaced with standard Ca^{2+}-containing medium. (*Middle*) Mezerein was added to a muscle strip incubated in the Ca^{2+}-free, 1 mM EGTA medium. The Ca^{2+}-free medium was replaced with Ca^{2+}-containing medium at 192 min. (*Bottom*) Mezerein was added to a muscle strip incubated in the Ca^{2+}-free, 1 mM EGTA medium. At 155 min, 20 mM BAY K 8644 was added. The Ca^{2+}-free medium was replaced with Ca^{2+}-containing medium at 190 min. Solid lines represent time periods when tissue strips were incubated in standard Ca^{2+}-containing buffer. Dashed lines represent periods when the incubation medium was switched to a Ca^{2+}-free, 1 mM EGTA Tyrode buffer.

Similarly, the data in Fig. 5 *Middle* and *Bottom* show that responses to mezerein or mezerein and BAY K 8644 were inhibited in muscle strips incubated in the absence of extracellular Ca^{2+}. Addition of extracellular Ca^{2+} resulted in a contractile response in both cases. However, muscle treated with BAY K 8644 as well as mezerein (Fig. 5 *Middle*) produced a contractile response that was faster and greater in magnitude than muscle treated with mezerein alone (compare Fig. 5 *Bottom* and *Middle*). Again, these responses could be reversed completely by removing extracellular Ca^{2+} (results not shown).

Effect of the Antagonist on Drug-Induced Responses. In all of the above experiments, 0.1 μM atropine sulfate was added to the tissue bath at least 5 min prior to the addition of specific pharmacologic agents to avoid the possibility that any acetylcholine released as a consequence of drug action might induce contraction. To demonstrate that the release of histamine was not responsible for the observed responses, experiments were repeated in the presence of 0.1 μM atropine sulfate and 1 μM diphenhydramine, an H_1 antagonist. The combined addition of ionomycin and mezerein or sequential addition of mezerein and BAY K 8644 had effects that were qualitatively unchanged by the presence of diphenhydramine. However, addition of this drug did result in a 35–45% decrease in the magnitude of all responses, including the carbachol-induced response.

DISCUSSION

The present results indicate that tracheal smooth muscle can be induced to contract by agents that activate the C-kinase. The effects of these agents can be blocked by removing extracellular Ca^{2+} (Fig. 5) or enhanced by simultaneous exposure of the muscle to drugs that either stimulate plasma membrane calcium flux and/or raise cytosolic free Ca^{2+} (Figs. 1–4). Figs. 1 and 2 show that the combined addition of either A23187 and PMA or mezerein and ionomycin induces a synergistic contractile response. This synergism is also demonstrable with the combined addition of the calcium channel agonist BAY K 8644 and C-kinase activators (Fig. 3). In the latter case, the response is completely reversible by removing extracellular Ca^{2+} (Fig. 5). Since C-kinase is an enzyme sensitive to diacylglycerol-like activators (e.g., PMA and mezerein) and Ca^{2+}, this synergism in the contractile response suggests the presence of a C-kinase-mediated contractile mechanism in smooth muscle.

It is important to emphasize that although these experiments were done on muscle strips that contain nerve endings and blood cells, it is unlikely that these results can be explained by drug-induced release of neurotransmitter (acetylcholine) or by slow endogenous release of histamine—acetylcholine and histamine being two known stimulators of tracheal smooth muscle contraction (24). All experiments were performed on tissues exposed to 0.1 μM atropine, a dose that has been reported to be sufficient to block the action of neurotransmitter released from nerve endings in response to K^+ depolarization in these tissues (6). Furthermore, when experiments were repeated on tissues exposed to atropine as well as diphenhydramine, an H_1 blocker, the responses were

qualitatively unchanged, although the magnitude of all responses, including that of the carbachol, was significantly reduced.

Finally, it is noteworthy that the contractile responses induced by various drug combinations were rapidly and completely reversed by addition of forskolin (Fig. 4), an agent shown to inhibit agonist-induced contractions. Likewise, these responses depend on extracellular Ca^{2+} (Fig. 5), analogous to carbachol-induced responses, especially during the sustained phase. The reversibility of these drug-induced contractile responses indicates that they are not a toxic, nonreversible result of the drugs used. In short, these pharmacological agents appear to mimic the postreceptor events involved in smooth muscle contraction by acting directly on the muscle itself. The different contractile responses elicited with agents that increase intracellular Ca^{2+} versus those that activate C-kinase, the synergism between these two classes of drugs resulting from their combined action on the muscle, as well as the dependency of these responses on extracellular Ca^{2+} strongly support the idea that receptor-mediated contraction of smooth muscle involves Ca^{2+} action at two different levels: (*i*) activation of calmodulin-dependent MLC kinase, which is responsible for the initial phase of the contractile response, and (*ii*) activation of C-kinase, which is responsible for the sustained phase of this response (17, 18).

This model is supported by a number of other lines of evidence. First, there is abundant C-kinase in smooth muscle (25). Second, in several types of smooth muscle, appropriate natural agonist activates the turnover of polyphosphoinositides (25–27), giving rise presumably to inositol trisphosphate and diacylglycerol in these tissues as they do in others (28, 29). Third, addition of inositol trisphosphate to saponin-permeabilized, calcium-loaded smooth muscle cells induces a mobilization of Ca^{2+} from an ATP-dependent, nonmitochondrial calcium pool (30) as it does in other tissues (14, 31–33). These various lines of evidence, previous data obtained from studies on vascular smooth muscle (21), and the present results all support the concept that the C-kinase branch of the calcium messenger system plays a key role in regulating the sustained phase of tracheal smooth muscle contraction (17). In this regard, it is noteworthy that the MLC (34), the MLC kinase (35), and the actin-binding proteins vinculin and filamin (36) are substrates for C-kinase *in vitro*. It is not yet known whether these proteins become phosphorylated in response to agonist or C-kinase activators *in situ*.

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