The Effects of Isoproterenol on Intracellular Calcium Concentration*

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β-Adrenergic agonist, isoproterenol (ISO), is a potent relaxant of tracheal smooth muscle and inhibits carbachol-induced contraction. The effect of ISO on intracellular free Ca2+ concentration ([Ca2+]i) was examined in bovine tracheal smooth muscle strips, employing aequorin as Ca2+ indicator. Surprisingly, 10 μM ISO induces a 5-fold increase in [Ca2+]i, which then gradually declines but still remains higher than basal after 1 h of stimulation. The ISO-induced increase in [Ca2+]i, is dose-dependent, and the ED50 is approximately 50 nM. The ISO-induced increase in [Ca2+]i, is inhibited by a β-receptor blocker, propranolol, not by an α-blocker, phentolamine. The ISO-induced rise in [Ca2+]i, is dependent on extracellular Ca2+. Forskolin, an adenylate cyclase activator, and vasoactive intestinal peptide, a receptor blocker, propranolol, not by an α-blocker, phentolamine, inhibit the rise in [Ca2+]i, suggesting that a rise in cyclic AMP concentration mediates this effect of ISO on [Ca2+]i. Pretreatment of muscle with 10 μM ISO inhibits both the initial Ca2+ transient and the contractile response induced by 0.3 μM carbachol. Conversely, in carbachol-pretreated muscle strips, addition of ISO causes a fall rather than a rise in [Ca2+]i, and an inhibition of contraction. These results indicate that ISO has effects on cellular Ca2+ metabolism at an earlier stage in bovine tracheal smooth muscle, that these effects are different in control and carbachol-pretreated muscle, and that the relaxing effect of ISO is not due solely to its effect on Ca2+ metabolism.

β-Adrenergic agonists induce relaxation of many types of smooth muscle including tracheal smooth muscle (1–3). The agonist is known to exert its relaxing effect through the production of cyclic AMP (4). Numerous studies have suggested that phosphorylation of the 20 kDa myosin light chain (MLC) by a Ca2+ and calmodulin-dependent enzyme, myosin light chain kinase, is an essential event in the initiation of agonist-induced smooth muscle contraction (5–7). Previous studies by Adelstein et al. (8, 9) showed that a cyclic AMP-dependent protein kinase phosphorylates purified MLC kinase and inhibits its activity by reducing the affinity of MLC kinase for a Ca2+-calmodulin complex. They proposed that this phosphorylation of MLC kinase by cyclic AMP-dependent protein kinase might be a mechanism of cyclic AMP-mediated relaxation of smooth muscle (10). However, Stull et al. (3, 11) examined the properties of MLC kinase in bovine tracheal smooth muscle in situ and found no change in its activation properties whether isolated from control muscle or isoproterenol (ISO)-treated muscle. They questioned the physiological role of MLC kinase phosphorylation in mediating relaxation of bovine tracheal smooth muscle by the β-adrenergic agonist. They have suggested that cyclic AMP-dependent effects on cellular Ca2+ metabolism may be more important in mediating relaxation. In fact, cyclic AMP has been reported to influence several aspects of cellular Ca2+ metabolism in other types of smooth muscle including a stimulation of Ca2+ extrusion from cells (12, 13), an inhibition of Ca2+ influx into cells (14), and stimulation of Ca2+ uptake into intracellular storage sites (15–17).

We have recently demonstrated that addition of carbachol induces an increase in intracellular free Ca2+ concentration ([Ca2+]i) in bovine tracheal smooth muscle strips using the photoprotein, aequorin, as calcium indicator (18); a method developed by Morgan and Morgan (19) for studies in vascular smooth muscle. In the present study, to further explore the mechanism of cyclic AMP-induced relaxation of tracheal smooth muscle, we have measured changes in [Ca2+]i, in response to the β-adrenergic agonist, ISO, in control and carbachol-pretreated tracheal muscle strips. The results indicate that ISO alone induces a sustained increase in [Ca2+]i, but causes a fall in [Ca2+]i, when carbachol is also present.

EXPERIMENTAL PROCEDURES

Preparation and Aequorin-loading of Bovine Tracheal Smooth Muscle Strips—Calf tracheae were obtained from a local abattoir and transported at 4 °C in a modified Tyrode solution with the following composition: 137 mM NaCl, 2 mM KCl, 0.3 mM NaH2PO4, 1.0 mM MgCl2, 2.25 mM CaCl2, 5.5 mM glucose, and 11.9 mM NaHCO3. Trachealis smooth muscle strips were dissected as described elsewhere (20). A muscle strip of 1–1.5 cm length was fixed on plastic support and loaded with aequorin by procedures employing a modification (21) of the method of Morgan and Morgan (19). Briefly, this procedure consists of incubating tracheal smooth muscle strips in a series of four solutions at 4 °C for 30–120 min. The first step is three 10-min incubations in the following solution: 120 mM K glutamate, 20 mM TES, 10 mM EGTA, 5 mM adenosine 5'-triphosphate (NaATP), 2 mM MgCl2, pH 7.1. The second step is an hour incubation in the following solution: 120 mM K glutamate, 20 mM TES, 0.1 mM EGTA, 2 mM MgCl2, 50 μg/ml aequorin, pH 7.1. The third step is a 2-h incubation in the following solution: 120 mM K glutamate, 20 mM TES, 0.1 mM EGTA, 5 mM NaATP, 10 mM MgCl2, pH 7.1. The fourth step is a 2-h incubation at 4 °C in the following solution: 137 mM NaCl, 2 mM KCl, 0.3 mM NaH2PO4, 12.5 mM MgCl2, and 5.5 mM glucose aerated with 95% O2, 5% CO2. Re-addition of calcium was done by gradually increasing a Ca2+ concentration up to 1.8 mM over a 30-min period at room temperature in a modified...
Temperature in Dulbecco's modified Eagle's minimal essential medium preparation, luminescence was expressed as a ratio (L/LBA\textsubscript{ASAL}) to measurement of aequorin luminescence has been described elsewhere (21, 22). Overnight recovery was carried out by incubating strips at room temperature in Tyrode solution initially without Ca\textsuperscript{2+} and gassed with 95% O\textsubscript{2}, 5% CO\textsubscript{2}. The buffers containing high K concentrations were preincubated in the Tyrode solution gassed with 95% O\textsubscript{2}, 5% CO\textsubscript{2}. When aequorin luminescence is calibrated for the absolute value of [Ca\textsuperscript{2+}], the values of 80 ± 50 nM (mean ± S.D., n = 9) for the unstimulated base-line value and 370 ± 80 nM (n = 7) for the peak value are obtained.

The dose-dependent response of luminescence as a function of ISO concentration is shown in Fig. 2B. The ISO-induced contractile responses seen in aequorin-loaded muscle strips, it induces a rapid increase in luminescence. There is a rapid and sustained contractile response (Fig. 1A). These contractile responses seen in aequorin-loaded muscle strips are qualitatively similar to those obtained using non-aequorin loaded fresh tracheal smooth muscle strips (11, 20).

**RESULTS**

Effect of ISO on Carbachol-induced Contraction of Bovine Tracheal Smooth Muscle—The effect of ISO on carbachol-induced contraction of aequorin-loaded tracheal smooth muscle strips is shown in Fig. 1. Addition of 0.3 \textmu M carbachol induces a rapid and sustained contractile response (Fig. 1A). When 10 \textmu M ISO is added to muscle strips precontracted with 0.3 \textmu M carbachol, ISO brings about a rapid and complete reversal of the contraction (Fig. 1B). Pretreatment of muscle strips with 10 \textmu M ISO completely inhibits the contractile response to subsequently added carbachol (Fig. 1C). These contractile responses in aequorin-loaded muscle strips are qualitatively similar to those obtained using non-aequorin loaded fresh tracheal smooth muscle strips (11, 20).

Effect of ISO on [Ca\textsuperscript{2+}]\textsubscript{i} in Bovine Tracheal Smooth Muscle—When 10 \textmu M ISO is added to aequorin-loaded tracheal smooth muscle strips, it induces a rapid increase in luminescence (Fig. 2A). Aequorin luminescence reaches a peak within 4–5 min, then gradually declines but is still 4- to 5-fold higher than the unstimulated base-line value after 60 min of stimulation. When aequorin luminescence is calibrated for the absolute value of [Ca\textsuperscript{2+}]\textsubscript{i}, the values of 80 ± 50 nM (mean ± S.D., n = 9) for the unstimulated base-line value and 370 ± 80 nM (n = 7) for the peak value are obtained.

The dose-dependent response of luminescence as a function of ISO concentration is shown in Fig. 2B. The ISO-induced

![Fig. 1. The effect of isoproterenol on carbachol-induced contraction. A, the sustained contraction induced by 0.3 \textmu M carbachol, B, the reversal of 0.3 \textmu M carbachol-induced contraction by 10 \textmu M ISO, C, the inhibition of 0.3 \textmu M carbachol-induced contraction by pretreatment with 10 \textmu M ISO.](image)

![Fig. 2. The effect of isoproterenol on intracellular free Ca\textsuperscript{2+} concentration. A, aequorin-loaded tracheal smooth muscle strips were stimulated with 10 \textmu M ISO for 60 min. Aequorin luminescence is expressed as a ratio of luminescence at each time point to the unstimulated base-line luminescence as described under “Experimental Procedures”; BG on the y axis indicates background current. B, aequorin-loaded tracheal smooth muscle strips were stimulated with various concentrations of ISO (10\textsuperscript{-6} M to 3 × 10\textsuperscript{-5} M). The amplitude of peak luminescence is expressed as percent of the maximal response. The maximal response is 6.5-fold increase of the base-line luminescence at 30 \textmu M ISO. Each point is a mean ± S.E. of four determinations.](image)
increase in luminescence increases with increasing doses of ISO. A concentration as low as 1 nM causes a significant increase in luminescence. The half-maximal effect is observed at approximately 50 nM ISO. This dose-response relationship is very similar to that for ISO-induced relaxation of carbachol-induced contraction reported by others (11).

The effects of various adrenergic receptor inhibitors on the ISO-induced increase in $[\text{Ca}^{2+}]$, are shown in Fig. 3. Propranolol, a $\beta$-adrenergic antagonist, abolishes the ISO-induced increase in $[\text{Ca}^{2+}]$. (Fig. 3, A and B). Phenotamine, an $\alpha$-adrenergic antagonist, does not inhibit the effect of ISO (Fig. 3C). These results indicate that the ISO-induced increase in $[\text{Ca}^{2+}]$, is a $\beta$-adrenergic receptor-mediated event. When atropine, a muscarinic cholinergic antagonist, at doses up to 1 $\mu$M, is added to tracheal smooth muscle, the effect of ISO on $[\text{Ca}^{2+}]$, is not inhibited. This finding indicates that the ISO-induced increase in $[\text{Ca}^{2+}]$, is not mediated by release of endogenous acetylcholine from nerve terminals.

**Effect of Forskolin and Vasoactive Intestinal Peptide on $[\text{Ca}^{2+}]$, in Bovine Tracheal Smooth Muscle—**Because $\beta$-adrenergic agents are known to stimulate adenylate cyclase and induce an increase in cyclic AMP production, the effect of forskolin, a more direct activator of adenylate cyclase, on $[\text{Ca}^{2+}]$, was examined in bovine tracheal smooth muscle. As shown in Fig. 4A, addition of 25 $\mu$M forskolin produces an increase in $[\text{Ca}^{2+}]$, which displays a similar temporal pattern and a similar magnitude as the increase induced by 10 $\mu$M ISO (compare Fig. 4 with Fig. 2A). This dose of forskolin also possesses a potent relaxing effect and causes a nearly complete inhibition of the effect of 0.3 $\mu$M carbachol on contraction (20). Moreover, vasoactive intestinal peptide, which is known to relax tracheal smooth muscle through a cyclic AMP-mediated mechanism (27), also induces an increase in $[\text{Ca}^{2+}]$, although the extent of both relaxation and rise in $[\text{Ca}^{2+}]$, induced by this agent is smaller than that caused by ISO or forskolin (Fig. 4B). These results suggest that the ISO-induced increase in $[\text{Ca}^{2+}]$, may be mediated by a rise in cyclic AMP content. However, the cyclic AMP analog, dibutyryl cyclic AMP does not induce an increase in $[\text{Ca}^{2+}]$, when this agent is used in doses up to 1 mM. Since this analog causes almost no relaxation in carbachol-stimulated tracheal smooth muscle over the same dose range as employed in the aequorin experiments, the inability of this cyclic AMP analog to induce an increase in $[\text{Ca}^{2+}]$, appears to be related to the inability of this analog to penetrate into tracheal smooth muscle cells in a sufficient concentration to be effective.

**Effect of Extracellular $\text{Ca}^{2+}$ Removal on ISO-induced Increase in $[\text{Ca}^{2+}]$.**—In order to determine the origin of the $\text{Ca}^{2+}$ responsible for the ISO-induced rise in $[\text{Ca}^{2+}]$, the effect of the removal of extracellular $\text{Ca}^{2+}$ on ISO action was examined. As shown in Fig. 5A, when extracellular $\text{Ca}^{2+}$ is removed from smooth muscle strips stimulated with 10 $\mu$M ISO, the $[\text{Ca}^{2+}]$, declines to a value below the unstimulated base-line value. When $\text{Ca}^{2+}$ is reintroduced into the buffer, a rapid return of $[\text{Ca}^{2+}]$, to the original higher level is seen (data not shown). In smooth muscle strips which have been perfused with a $\text{Ca}^{2+}$-free buffer containing 2 mM EGTA for 20 min and then stimulated with 10 $\mu$M ISO.

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**Fig. 3.** Effect of various receptor blockers on isoproterenol-induced increase in intracellular free $\text{Ca}^{2+}$ concentration. Aequorin-loaded tracheal smooth muscle strips were treated with ISO and propranolol or phentolamine in the order indicated in the figure.
in the presence of Ca$^{2+}$ (Fig. 5B). Under the same Ca$^{2+}$-free condition, a lower dose of ISO (0.1 μM) does not induce any increase in [Ca$^{2+}$]i. Likewise, the rise in [Ca$^{2+}$]i, induced by 25 μM forskolin is nearly completely inhibited when the muscle strip is incubated in a Ca$^{2+}$-free medium (see Fig. 7C). In contrast, carbachol or caffeine, which are known to mobilize intracellular Ca$^{2+}$, still induces an increase in [Ca$^{2+}$]i, under these same Ca$^{2+}$-free conditions (see Fig. 7). These findings suggest that the ISO-induced rise in [Ca$^{2+}$]i is due largely to the stimulation of Ca$^{2+}$ influx across the plasma membrane.

Nimodipine, a dihydropyridine Ca$^{2+}$-channel antagonist, does not inhibit the ISO- (or forskolin-)induced increase in [Ca$^{2+}$]i, at doses up to 5 μM. These results are in contrast to the inhibitory effect of nimodipine on the high K+-induced increase in [Ca$^{2+}$]i, we previously observed in tracheal smooth muscle.²

**Effect of ISO on Carbachol-induced Initial Ca$^{2+}$ Transients—**As shown in Fig. 6A, carbachol induces a Ca$^{2+}$ transient followed by a lower sustained increase in [Ca$^{2+}$]i. When 0.3 μM carbachol is added to smooth muscle strips prestimulated with 10 μM ISO, no carbachol-induced Ca$^{2+}$ transient is seen (Fig. 6B). Rather, a fall in [Ca$^{2+}$]i, to a value only slightly greater than basal is seen. Likewise, pretreatment with 25 μM forskolin inhibits the carbachol-induced Ca$^{2+}$ transient (Fig. 6C).

This inhibition of the carbachol-induced Ca$^{2+}$ transient by ISO becomes even clearer when the muscle is incubated in the Ca$^{2+}$-free medium. As shown in Fig. 7A, 0.3 μM carbachol elicits a transient increase in [Ca$^{2+}$]i, in the absence of extra-

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² Y. Takuwa, N. Takuwa, and H. Rasmussen, unpublished data.

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**FIG. 6.** Effect of isoproterenol and forskolin on carbachol-induced Ca$^{2+}$ transients. 0.3 μM carbachol was added to a control strip (A), ISO-pretreated strip (B), or forskolin-pretreated strip (C).
whether or not the sequence of addition of ISO and carbachol was important in determining the ultimate value of $[Ca^{2+}]_i$.

Induced by Carbachol-The last point to be examined was the inhibitory effect on ISO-induced increases in cyclic AMP. Conversely, pretreatment of muscle strips with DPB does not have such an inhibitory effect on ISO-induced increases in cyclic AMP. However, when any of three agents, known to stimulate adenylate cyclase in this tissue, is employed, a sustained rise in $[Ca^{2+}]_i$ is observed (Figs. 2-6 and 8-10). This rise is approximately equivalent to that produced by 80 mM extracellular K+, but the consequences are quite different (28, 29). Treatment with high K+ leads to a contraction and the phosphorylation of the 20-kDa light chains of myosin, but treatment with ISO leads to neither (28, 29).

In view of this striking difference, the possibility that aequorin might be detecting $Ca^{2+}$ signals in more than one subcellular compartment was considered. For example, it might be possible that in addition to being loaded into the cytosolic compartment, this indicator might also be incorpo-

**TABLE I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cyclic AMP pM</th>
<th>$p$ value as compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>5.0 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>10 μM ISO</td>
<td>16.4 ± 2.5</td>
<td>$p &lt; 0.005$</td>
</tr>
<tr>
<td>0.3 μM carbachol</td>
<td>6.1 ± 0.7</td>
<td>0.05 &lt; $p &lt; 0.10$</td>
</tr>
<tr>
<td>10 μM ISO</td>
<td>13.3 ± 3.0</td>
<td>$p &lt; 0.005$</td>
</tr>
<tr>
<td>0.8 μM DPB + 10 μM ISO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

FIG. 9. Effect of 12-deoxyphorbol 13-isobutyrate on isoproterenol-induced increase in $[Ca^{2+}]_i$. A, a strip was first treated with 10 μM ISO and then with 800 nM DPB. B, a strip was first treated with 800 nM DPB and then with 10 μM ISO.

Effect of carbachol and 12-deoxyphorbol 13-isobutyrate on isoproterenol-induced increases in cyclic AMP in bovine tracheal smooth muscle

Strips of bovine tracheal smooth muscle were preincubated with or without either carbachol or DPB at 37°C for 40 min, then stimulated with ISO for 5 min. The reaction was terminated by quick freezing in liquid N₂ and extraction and measurement of cyclic AMP was carried out as described under "Experimental Procedures." Values are means ± S.D. of three determinations.

As shown in Figs. 6 and 8, addition of carbachol to ISO-preincubated muscle led to a fall in $[Ca^{2+}]_i$. As shown in Fig. 10A, when 10 μM ISO is added to muscle strips pretreated with 0.3 μM carbachol, a considerable reduction in aequorin luminescence of the plateau phase (81 ± 16% (n = 6)) is also seen. The same dose of ISO consistently induces a complete relaxation of muscle contracted with 0.3 μM carbachol (Fig. 10B). Furthermore, when propranolol is added following the addition of ISO, the $[Ca^{2+}]_i$ goes up to the original level induced by carbachol (Fig. 10A). These results indicate that ISO causes a reduction in the plateau phase of elevated $[Ca^{2+}]_i$; induced by carbachol and that ISO fails to induce an increase in $[Ca^{2+}]_i$ in the presence of carbachol. As shown in Fig. 10B, forskolin has a similar effect on the carbachol-induced increase in $[Ca^{2+}]_i$. These results indicate that regardless of the sequence of addition, combined carbachol and ISO lead to the same change in the plateau value of $[Ca^{2+}]_i$; a very slight increase in $[Ca^{2+}]_i$.

DISCUSSION

The present results were quite unexpected. Before beginning these experiments, we had thought, as Stull et al. (11) suggested, that a rise in the cyclic AMP concentration in tracheal smooth muscle would lead to a fall in $[Ca^{2+}]_i$. However, when any of three agents, known to stimulate adenylate cyclase in this tissue, is employed, a sustained rise in $[Ca^{2+}]_i$ is observed (Figs. 2-6 and 8-10). This rise is approximately equivalent to that produced by 80 mM extracellular K+, but the consequences are quite different (28, 29). Treatment with high K+ leads to a contraction and the phosphorylation of the 20-kDa light chains of myosin, but treatment with ISO leads to neither (28, 29).

In view of this striking difference, the possibility that aequorin might be detecting $Ca^{2+}$ signals in more than one subcellular compartment was considered. For example, it might be possible that in addition to being loaded into the cytosolic compartment, this indicator might also be incorpo-
rated into the sarcoplasmic reticulum. If so, then addition of isoproterenol could lead to an uptake of Ca\(^{2+}\) into this compartment, and hence an increase in [Ca\(^{2+}\)]. This possibility seems an unlikely one based on several lines of indirect evidence: 1) no such evidence for such a distribution of aequorin has been found in other tissues; 2) if the basal [Ca\(^{2+}\)] was due to a composite signal from these two subcellular compartments and the [Ca\(^{2+}\)] in the sarcoplasmic reticulum was higher than in the cytosol, then because of the nature of the dose-response curve for Ca\(^{2+}\) versus luminescence, this signal would provide a disproportionate value to this basal value, and upon activation of the cell by carbachol, a fall in the [Ca\(^{2+}\)] within the sarcoplasmic reticulum should counterbalance the rise in [Ca\(^{2+}\)] seen in the cytosol, and 3) if the sarcoplasmic reticulum has a higher Ca\(^{2+}\) than that in the cytosol, then during the overnight recovery period after aequorin loading but before measurement of [Ca\(^{2+}\)], the aequorin in the sarcoplasmic reticulum compartment would be expended; 4) more directly, in preliminary experiments in which the plasma membrane of an aequorin-loaded muscle strip was made permeable to Ca\(^{2+}\) by treatment with staphylococcus α-toxin (30), ISO caused no increase in aequorin luminescence. Under these conditions [Ca\(^{2+}\)], is a direct function of the extracellular [Ca\(^{2+}\)]. In the succeeding discussion, we have assumed that the changes in [Ca\(^{2+}\)] induced by isoproterenol are occurring in the same subcellular compartment as the changes induced by carbachol.

The present study shows clearly that a rise in cyclic AMP concentration in bovine tracheal smooth muscle is associated with an increase in [Ca\(^{2+}\)], in the absence of cholinergic activation and not a decrease as predicted. The facts that both ISO and forskolin induce similar changes in [Ca\(^{2+}\)], and that propanolol but not phentolamine inhibits the effect of isoproterenol argue strongly that the observed changes in [Ca\(^{2+}\)], are secondary to an increase in cyclic AMP concentration. Furthermore, the results shown in Figs. 5 and 7 indicate that the source of the Ca\(^{2+}\) for the increase in [Ca\(^{2+}\)], is largely the extracellular pool. This finding leads to the conclusion that one effect of an increase in cyclic AMP concentration in tracheal smooth muscle is an increase in Ca\(^{2+}\) influx rate across the plasma membrane via a channel which is not inhibited by dihydropyridine Ca\(^{2+}\)-antagonists. An increase in [Ca\(^{2+}\)], induced by ISO, forskolin, or adenosine has been reported in ferret portal vein strips, employing aequorin as a Ca\(^{2+}\) indicator (31, 32). However, the effect was much smaller than that found in tracheal smooth muscle in the present study and the pool of mobilized Ca\(^{2+}\) was not identified. We also observed a similar small effect of forskolin on [Ca\(^{2+}\)], in aequorin-loaded rabbit aortic strips.  

Such a cyclic AMP-dependent enhancement of Ca\(^{2+}\) influx across the plasma membrane has been reported in skeletal muscle (33), cardiac myocytes (34), hepatocytes (35), certain neurons (36), and other cells (37). What is unusual in the case of tracheal smooth muscle is that the resulting increase in [Ca\(^{2+}\)], is of such a great magnitude as that induced by 80 mM K\(^+\), but the physiologic consequences are quite different. In the case of ISO or forskolin, there is no increase in muscle tension, whereas with high K\(^+\) there is a significant and sustained increase in muscle tension (28). An increase in phosphorylation of MLC is seen with high K\(^+\), but not with ISO stimulation (28, 29). Hence, one must conclude that an additional effect of cyclic AMP (other than changing [Ca\(^{2+}\)]), operates and prevents the rise in [Ca\(^{2+}\)], from activating MLC kinase (11). This effect must either be that of preventing MLC kinase activation thereby inhibiting the phosphorylation of MLC; or that of activating the phosphoprotein phosphatase responsible for the dephosphorylation of phosphorylated MLC. Even so, such an effect cannot account for all of the actions of cyclic AMP on cell function.

When tracheal muscle strips are exposed to 0.3 μM carbachol, there is a very rapid and transient rise in [Ca\(^{2+}\)], which declines to a sustained plateau (Fig. 6). This plateau is significantly greater than the base-line value but less than the plateau of [Ca\(^{2+}\)], induced by 10 μM ISO (Fig. 8). Nevertheless, the subsequent addition of ISO (after carbachol) leads to a decrease in [Ca\(^{2+}\)], to a value only slightly greater than basal, and not to an increase as seen in control muscle (Fig. 10). Conversely, if 0.3 μM carbachol is added to a muscle strip exposed to 10 μM ISO for 20 min, carbachol does not induce any significant initial increase in [Ca\(^{2+}\)], but rather, after a brief time delay (3 min), a fall in [Ca\(^{2+}\)], to a value only slightly greater than basal is observed (Figs. 6 and 8). These results mean that the subsequent addition of ISO causes a carbachol-induced [Ca\(^{2+}\)], plateau to decrease, and the subsequent addition of carbachol causes an ISO-induced [Ca\(^{2+}\)], plateau to decrease to reach in each case a comparable [Ca\(^{2+}\)]. It is particularly noteworthy that addition of 800 nM DPN, an activator of C-kinase, also causes a fall in [Ca\(^{2+}\)], in ISO-treated muscle, and inhibits the effect of ISO on [Ca\(^{2+}\)], if added to a control muscle strip prior to the addition of ISO (Fig. 9) .

These results indicate that there is a complex relationship between the Ca\(^{2+}\) and cyclic AMP messenger systems in this tissue. To begin to understand these relationships, the mechanisms by which ISO alters the response of the tissue to carbachol will be considered first, and then the mechanism by which carbachol alters the response to ISO will be discussed.

The most striking effect of ISO (a high dose) pretreatment is that it totally inhibits the carbachol-induced [Ca\(^{2+}\)], transient. This inhibition is seen in muscle strips incubated either in normal Ca\(^{2+}\)-containing media, or in media containing no Ca\(^{2+}\) but EGTA (Figs. 6 and 7). Hence it is not due to the ISO-induced change in [Ca\(^{2+}\)], because this change is not seen after ISO treatment of strips incubated in Ca\(^{2+}\)-free media. However, the fact that caffeine still induces an increase in [Ca\(^{2+}\)], in such strips argues that the failure of carbachol to induce a [Ca\(^{2+}\)], transient is not due to a depletion of the intracellular Ca\(^{2+}\) pool since we showed that carbachol and caffeine mobilize Ca\(^{2+}\) from the same intracellular pool (21).

These results indicate that ISO, acting via cyclic AMP, inhibits the carbachol-activated Ca\(^{2+}\), transient either by inhibiting the generation of inositol 1,4,5-trisphosphate, the intracellular messenger responsible for Ca\(^{2+}\) mobilization (38, 39), or by inhibiting the action of inositol 1,4,5-trisphosphate. There is no evidence from studies in other systems to support the latter suggestion, but studies have shown that in platelets, an increase in cyclic AMP concentration leads to an inhibition of agonist-induced phospholipase C activation and inositol 1,4,5-trisphosphate production (40–42).

It is of equal interest to consider the effects of carbachol in ISO-pretreated tissues. When carbachol acts in ISO-pretreated tissue, it not only is ineffective in inducing a rise in [Ca\(^{2+}\)], it actually causes a decrease in the plateau phase of the increase in [Ca\(^{2+}\)], induced by ISO (Figs. 6 and 8). The most likely explanation for this effect of carbachol on [Ca\(^{2+}\)], is a carbachol-induced inhibition of cyclic AMP production. As shown by Lohmann et al. (43) and Table I in the present study, when 10 μM ISO is added to control tracheal smooth muscle strips, a 3-fold increase in cyclic AMP concentration is seen, but when 10 μM ISO is added to carbachol-pretreated muscle, there is only a very small increase (about 20%) in
Cyclic AMP concentration. Preliminary data from our laboratory show that carbachol inhibits ISO-stimulated adenylyl cyclase activity in isolated membranes from tracheal muscle. On the other hand, DPB, a protein kinase C activator, inhibits the ISO-induced rise in $[Ca^{2+}]_{i}$ (Fig. 9), but not the ISO-induced rise in cyclic AMP (Table I). Activation of protein kinase C leads to the inhibition of the ISO-induced increase in $Ca^{2+}$ influx across the plasma membrane through its action on a site after cyclic AMP production. Thus, it appears that carbachol inhibits adenylyl cyclase activation, and this leads to a decrease in cyclic AMP concentration and hence a decrease in the cyclic AMP-mediated increase in $Ca^{2+}$ influx rate. Furthermore, if the inhibition by ISO of the carbachol-induced activation of phospholipase C is not complete, protein kinase C activation may also contribute to the carbachol-induced inhibition of the ISO-induced $Ca^{2+}$ influx by a more direct effect on the $Ca^{2+}$ influx pathway.

The present work clearly points to the need for additional experiments in order to fully characterize the various cellular effects that carbachol may have on smooth muscle tone. The results shown in Figs. 6, 8, and 10 point out in dramatic fashion that there is no simple correlation between $[Ca^{2+}]_{i}$ in smooth muscle cells and the tone which the muscle exhibits.

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REFERENCES


G. Kelley, unpublished data.