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The Effects of Isoproterenol on Adenosine Cyclic 3',5'-Monophosphate and Contractility in Isolated Smooth Muscle Cells

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SUMMARY

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Suspensions of individual smooth muscle cells were isolated by enzymatic digestion of stomach muscle of *Bufo marinus*. The role of cyclic 3',5'-AMP as a mediator of *beta* adrenergic-induced decreases in the contractility of smooth muscle was assessed by comparison of the time course and dose dependence for the effects of the *beta* adrenergic agent isoproterenol on both parameters. Drug-induced changes in the contractile state of isolated smooth muscle cells were determined utilizing a Coulter counter. The ability of isoproterenol to inhibit contractions produced by subsequent exposure to the excitatory agent carbamylcholine was used as an index of decreases in contractility. Exposure to isoproterenol for more than 5 sec was necessary to demonstrate significant inhibition of contraction. Maximal effects of isoproterenol were noted after exposure for 15 sec. Basal cyclic AMP levels, calculated from measurements of cyclic AMP content of cell suspensions and intracellular fluid volume, were 4.5 μM. Exposure to isoproterenol for as little as 2.5 sec produced a significant increase in cyclic AMP levels; maximal changes were noted after exposure for 5 sec or more. The changes in cyclic AMP and contractility exhibited similar dose-response curves, with Ed_{50} values of approximately 0.4 μM. Prior incubation of the cells with theophylline, cyclic AMP, or N^6,2'-*O*-dibutyryl cyclic AMP also inhibited contractions induced by carbamylcholine. These findings strongly support the hypothesis that decreases in the contractility of smooth muscle produced by *beta* adrenergic agents may be mediated by an increase in cyclic AMP. The Coulter counter results also reveal that individual cells are capable of responding to both cholinergic and adrenergic transmitters. Thus it is likely that integration of the cholinergic excitatory and adrenergic inhibitory input to the whole tissue takes place in part, if not entirely, in individual cells.

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INTRODUCTION

The hypothesis that an increase in cyclic 3',5'-AMP concentration may mediate the decreased contractility induced by cate-

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cholamines in smooth muscle was first suggested by the findings of Bueding *et al.* (1) that *beta* adrenergic agonists cause both an increase in cyclic AMP levels and relaxation in guinea pig taenia coli. Subsequent studies have correlated an increase in tissue cyclic AMP levels with decreases in contractility in smooth muscle produced by catecholamines as well as other agents (2–6). In order for such studies to provide strong support for the role of cyclic AMP in causing relaxation in response to *beta* adrenergic agents, it must be shown that (a) changes in cyclic AMP precede the decrease in contractility, and (b) the magnitude of the change in cyclic AMP exhibits a continuous dependence on the concentration of catecholamines over the range in which the contractile effect is also dose-dependent. The hypothesis may be strengthened if it can be shown that agents such as theophylline or dibutyryl cyclic AMP, whose actions in other systems mimic an increase in cyclic AMP, also produce decreases in contractility (7). In a few studies one or several of these criteria have been satisfied (2–5), but in many others the expected temporal and steady-state relationships between cyclic AMP levels and changes in contractility have not been found (8–11).

One problem in relating the time course and dose dependence of changes in cyclic AMP to changes in contractile state arises from the nature of the preparations studied. Intact smooth muscle strips are characterized by complex electrical and mechanical interconnections of muscle cells, significant diffusion barriers, and the presence of nerve and other cell types in addition to smooth muscle cells (12). These complexities make investigations of the relationships between biochemical and physiological changes difficult to interpret. It is not surprising that results of various studies on the effect of catecholamines on cyclic nucleotide metabolism and contractility in intact smooth muscle have been used both to support (1–6) and to disclaim (8–10) a role of cyclic nucleotides in the induced relaxation.

The development of methods for preparation of isolated smooth muscle cells (13, 14) and the ability to assess the contractile state of large numbers of these cells in suspension (15) prompted us to investigate the effects of the *beta* adrenergic agonist isoproterenol on the cyclic AMP content and contractile state of isolated smooth muscle cells. Since these cell suspensions consist of smooth muscle cells without diffusion barriers or complex interconnections, they represent an advantageous system in which to test the proposed role of cyclic AMP in mediating the relaxation induced by catecholamines.

METHODS

Suspensions of single isolated smooth muscle cells were prepared from the stomach muscularis of the toad *Bufo marinus* by enzymatic digestion with trypsin and collagenase, using the method of Singer and Fay (15). The contractile state of the cells in suspension was determined using a Coulter counter according to the technique described by Singer and Fay (15). In essence, the technique involves drawing the cells in suspension through an orifice across which a constant electric current is maintained. As the cells traverse the orifice, an electrical pulse is produced whose amplitude is related to cell diameter and whose duration is related to cell length. Relaxed cells, which are long and thin, produce a low-amplitude, long-duration pulse. Upon contraction the cells become shorter and wider and thus produce a pulse of higher amplitude and shorter duration. Previous experiments (15) showed that measurements of pulse height provided a more reproducible index of the contractile state of cells in suspension than did measurement of pulse duration. Determination of the effect of a given agent on contractile state of the cells is carried out as follows: (a) a suspension of cells is divided into a series of aliquots; (b) to all but one aliquot (the control) the agents under study are added, and after a set time the cells in all aliquots are fixed rapidly with either glutaraldehyde (2%) or acrolein (1%); (c) the fixative is removed by centrifuging the cell suspension at 200 $\times g$ for 10 min and resuspending the pellet in 0.9% NaCl; (d) cells from each aliquot

are then passed through a Coulter counter and a histogram of pulse amplitudes is generated for 10^4 particles. The histogram for each aliquot is compared with the control, and contraction (where a greater number of wider cells are present) is indicated by a shift in the pulse height histogram toward larger-amplitude pulses. The extent of contraction in a test aliquot relative to the control may be quantitated by calculating the increased percentage of larger-amplitude pulses generated by cells in the treated group relative to the control. As discussed by Singer and Fay (15), this measure of the shift in pulse height distribution may be used as a continuous index of the contractile state of isolated smooth muscle cells in suspension. This general approach may also be used to determine the effects of inhibitory agents, such as isoproterenol, that cause a decrease in the contractility of smooth muscle. Since the cells in suspension are already relaxed and do not spontaneously contract, the addition of inhibitory agents does not result in a shift in the pulse height histograms. Therefore the decrease in contractility must be determined indirectly by measuring either the relaxation of cells previously contracted by exposure to excitatory agents (true relaxation) or the inhibition of contractions after incubation with the inhibitory agent (decrease in the potential for contraction). The ability of isoproterenol to decrease contractility was apparent when either of these methods was used. However, the time course for the development of the effects of isoproterenol when relaxation was examined was very much slower than when the effects of isoproterenol were assessed as a change in potential for contraction.[2] The long time course for isoproterenol-induced relaxation of cells would bias the data in favor of the hypothesis that changes in cyclic AMP occur prior to changes in contractility. Therefore we determined the changes in potential for contraction by first incubating the cells with isoproterenol and then challenging them with a brief (6-sec) exposure to the excitatory agent, carbamylcholine (carbachol). The effects of isoproterenol could then be determined by comparison of the pulse height histograms generated by cells exposed to carbachol alone with histograms generated by cells exposed to isoproterenol prior to carbachol.

For measurement of the cyclic AMP content of the cell suspensions, aliquots of the cell suspension were sonicated (Heat Systems–Ultrasonics, Inc., model W-185) for 15 sec in the presence of 2% perchloric acid. The extracts were then buffered by the addition of Tris base (10 mM, final concentration) and neutralized by the addition of KOH. Excess K^+ was added as KCl to ensure complete precipitation of perchlorate ion. After centrifugation to remove the precipitated perchlorate, the cyclic AMP in the extract was adsorbed on a 3-ml column of Bio-Rad AG1-X8 (Cl^-) ion-exchange resin. The column was washed with 4 ml of Tris-HCl buffer (10 mM, pH 7.5) and with 6 ml of distilled water. Cyclic AMP was eluted with the addition of 8 ml of 0.06 N HCl, and the effluent was lyophilized. The dried extract was dissolved with water, and duplicate aliquots at two different dilutions were assayed for cyclic AMP by a competitive protein binding assay described by Gilman (16). Nonspecific interference in the assay was determined by treating the cell extracts with cyclic nucleotide phosphodiesterase purified from beef heart (Sigma). A trace (0.1 pmole) of cyclic [^{3}H]AMP was added to the cell suspension prior to sonication to allow correction for loss of nucleotide during the subsequent purification procedures. The following chemicals were used in these studies: *l*-isoproterenol hydrochloride (Pflaltz and Bauer), carbamylcholine chloride (carbachol) (Sigma), propranolol hydrochloride (Sigma), disodium adenosine cyclic 3′,5′-monophosphate (Sigma), N^6,2′-*O*-dibutyryl cyclic AMP (Sigma), theophylline (Eastman Kodak), adenosine (Sigma), and cyclic [^{3}H]AMP

[2] The $t_{1/2}$ for isoproterenol-induced relaxation was approximately 30 sec, whereas the inhibition of contractions developed more quickly ($t_{1/2}$ = 10 sec; see RESULTS). The slow relaxation is probably not due to the slow development of the effects of isoproterenol but more likely results from the lack of large forces to re-extend the cells following cessation of contractile activity (14).

(New England Nuclear).

The time required for mixing of drug after addition to the cell suspension was approximately 2 sec, as assessed by dye dilution. Complete disruption of all cells by sonication in the presence of perchloric acid required 1 sec, as assessed by microscopic observation.

The cyclic AMP content in aliquots of cell suspensions, as well as the magnitude of drug-induced changes in contractile state, was found to vary between cell preparations. This variability presumably reflects differences in the yield of intact and functional smooth muscle cells as well as biological variation. To minimize the contribution of this variation in assessing drug-induced changes in cyclic AMP content, and to allow pooling of data obtained from many experiments, the cyclic AMP content in unstimulated cells was normalized. This was achieved by multiplying all values obtained from a given experiment (both unstimulated and stimulated cells) by a factor such that the cyclic AMP content of aliquots of unstimulated cells was made equal to the mean value obtained for all experiments. Data obtained from all experiments were then pooled and the effects of isoproterenol were analyzed by Student's *t*-test. Data obtained for isoproterenol-induced inhibition of contractions were also normalized by expressing the submaximal responses as a percentage of the maximal response achieved in each experiment. Data obtained from all experiments were then pooled and the effects of isoproterenol were analyzed by Student's *t*-test. ED_{50} values and associated 95% confidence limits were calculated by the method of Spearman and Klaiber (see ref. 17).

In several experiments the absolute concentration of cyclic AMP in the isolated cells was calculated by determining the volume of intracellular water per milliliter of cell suspension. This was done by collecting the cells in suspension using the silicone oil centrifugation method of Manno and Schacter (18). Microscopic examination revealed that only smooth muscle cells pass through the silicone oil layer during centrifugation. Prior to centrifugation, [^{3}H]inulin and [^{14}C]urea (both obtained from New England Nuclear) were added to the cell suspension to measure, respectively, the trapped extracellular fluid volume and the total (intracellular plus extracellular) volume of fluid passing through the silicone oil layer. The trapped extracellular fluid volume was calculated by dividing the amount of [^{3}H]inulin collected below the oil layer by the [^{3}H]inulin concentration in the original cell suspension; the combined intracellular plus extracellular volume collected below the oil was calculated by dividing the amount of [^{14}C]urea collected below the oil layer by the [^{14}C]urea concentration in the original cell suspension. The difference between the trapped extracellular fluid volume and the combined extracellular plus intracellular volumes was taken as a measure of the volume of intracellular fluid passing through the silicone oil layer. In all these experiments sufficient time (10 min) was allowed for the [^{14}C]urea to distribute itself throughout the extracellular and intracellular compartments prior to centrifugation.

RESULTS

Figure 1 illustrates the pulse height distribution for three aliquots of cell suspension. The left-hand panel compares the distribution for an aliquot of cell suspension left untreated ("Control," shaded area) with the distribution obtained with an aliquot exposed to carbachol at a concentration of 0.55 μM for 15 sec. The distribution obtained for the aliquot exposed to carbachol shows a shift in the pulse heights toward those with higher amplitude. Since contracted cells produce pulses of greater height, the carbachol-induced shift presumably reflects contractions of the cells. This interpretation was supported by direct microscopic observation (Fig. 2), which revealed that in the carbachol-treated aliquot there was a marked increase in the proportion of contracted cells relative to the control. As noted in METHODS, exposure to isoproterenol alone failed to produce any consistent changes in pulse height distributions, presumably because the cells were already relaxed. Therefore the ability of isoproterenol to

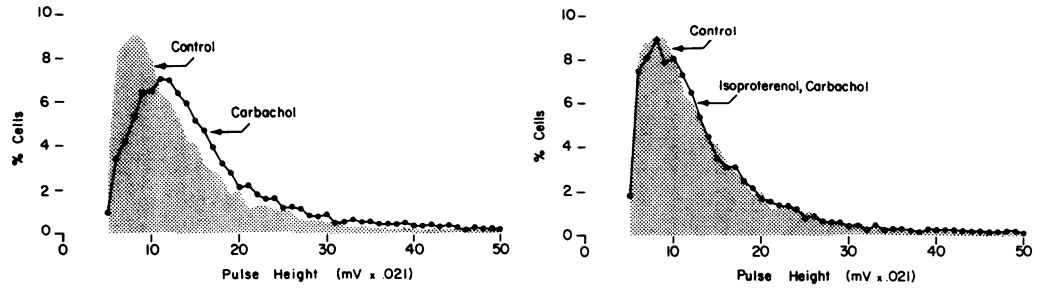

FIG. 1. *Pulse height histograms generated from isolated smooth muscle cells after exposure to carbachol and isoproterenol*

Prior to fixation the "Carbachol" aliquot was exposed to carbachol (0.55 μM) for 15 sec. The "Isoproterenol, Carbachol" aliquot was incubated with isoproterenol (0.8 μM) for 15 sec prior to exposure to carbachol (0.55 μM for 15 sec). The total period of exposure of this aliquot to isoproterenol was thus 30 sec. The "Control" aliquot was left untreated prior to fixation. Note that carbachol induced a shift in the pulse height histogram such that a greater percentage of large-amplitude pulses was seen relative to the control. Prior incubation with isoproterenol almost totally prevented the shift expected for a 15-sec exposure to carbachol.

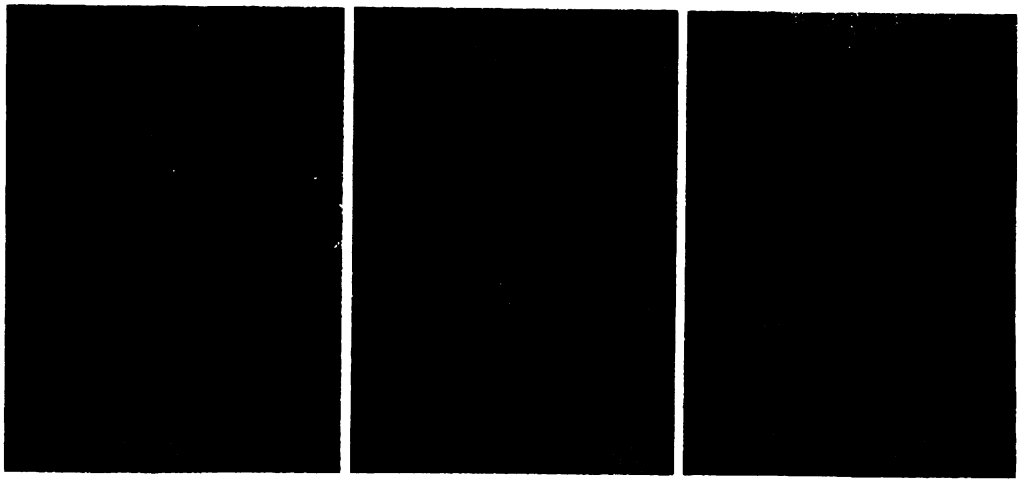

FIG. 2. *Photomicrographs of representative groups of smooth muscle cells from three aliquots of a single cell suspension*

The aliquots photographed were those on which the pulse height analyses in Fig. 1 were obtained. One aliquot was untreated ("Control") prior to fixation, another was exposed to carbachol (CbCl) prior to fixation, and the isoproterenol and carbachol group was incubated with isoproterenol (Iso) before treatment with carbachol. Phase contrast, × 62.

inhibit contractions in response to carbachol was used as an index of the effects of the catecholamine on contractility. The right-hand panel of Fig. 1 compares the pulse height distributions of untreated cells ("Control," shaded area) with that for cells exposed to 0.55 μM isoproterenol for 15 sec prior to exposure to carbachol (0.55 μM) for 15 sec. Exposure to isoproterenol decreased the potential for contraction, since carbachol failed to shift the pulse height distributions toward those with higher amplitudes. This interpretation was supported by direct microscopic observations (Fig. 2), which revealed that in suspensions exposed to isoproterenol, carbachol failed to increase the proportion of contracted cells. The effects of isoproterenol were quantitated by comparing the shifts in pulse height distribution produced by carbachol alone with the shifts produced in aliquots previously incubated with isoproterenol. For example, in the experiment shown in Fig. 1, carbachol

exposure shifted the pulse height distribution such that there were 20.2% more larger-amplitude pulses than in the control; in the aliquots of cells previously incubated with isoproterenol, carbachol exposure produced a much smaller shift (2.6% more of the larger-amplitude pulses than in the control). Therefore the exposure to isoproterenol produced 79% inhibition of the response to carbachol.

The time course for the effects of isoproterenol on the ability of cells to respond to carbachol is presented in Fig. 3, lower panel. Exposure of cells to isoproterenol for at least 5 sec was necessary before any inhibition of the response to carbachol was observed. Maximal inhibition was obtained with a 15-sec exposure to isoproterenol. As judged from this curve, the time necessary to achieve the half-maximal re-

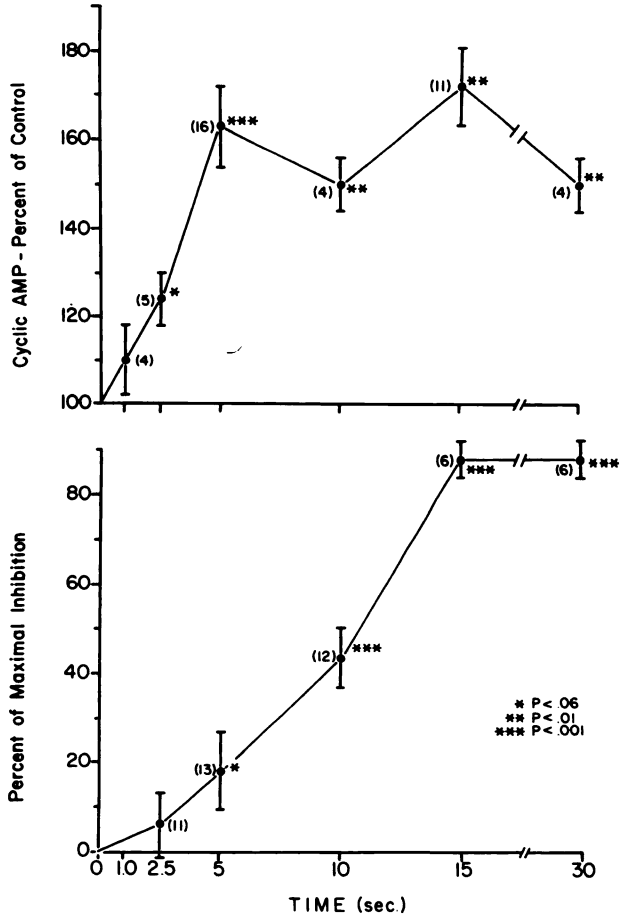

FIG. 3. *Time course for effects of isoproterenol (10 μM) on cyclic AMP levels (upper) and contractility (lower)*

In each experiment cyclic AMP levels were expressed relative to the levels in unstimulated cells. Each point represents the mean ± standard error calculated for data obtained from the number of experiments indicated in parentheses. The effects of isoproterenol on contractility were assessed indirectly as inhibition of carbachol-induced contraction (5.5 μM for 6 sec). Cells were exposed to isoproterenol for the length of time indicated on the abscissa. The maximum inhibition of the response to carbachol was always observed after exposure to isoproterenol for 15–60 sec. The extent of inhibition achieved at all times was expressed relative to the maximum achieved in each experiment. Note in the lower panel that the changes in cyclic AMP levels appeared to reach a maximum at 5 sec, a time when inhibition of contraction was just apparent. Furthermore, cyclic AMP levels were significantly elevated at 2.5 sec, although no significant inhibition of contraction was detectable.

sponse to isoproterenol ($t_{1/2}$) was 10 sec.

Suspensions of isolated smooth muscle cells were found to contain cyclic AMP. Resting levels of the nucleotide were calculated to be between 4 and 5 μM, assuming uniform distribution of cyclic AMP throughout the cell water (Table 1). The time course for the effects of isoproterenol on cyclic AMP levels in cell suspensions is shown in Fig. 3, upper panel. Within 2.5 sec after addition of isoproterenol, cyclic AMP levels were found to increase significantly (24% $p = 0.03$). Moreover, the increase in cyclic AMP levels appeared to reach nearly maximal levels within 5 sec and remained elevated for at least 60 sec. As judged from this curve, the $t_{1/2}$ for changes in cyclic AMP is approximately 3 sec. Comparison of the upper and lower panels in Fig. 3 indicates that isoproterenol induced a maximal change in cyclic AMP level at least 10 sec prior to maximal changes in contractility. Furthermore, isoproterenol caused a significant increase in cyclic AMP levels 2.5 sec before effects on contractility could be detected.

As a further test of a possible cause-and-effect relationship between changes in cyclic AMP and contractility, experiments were performed to compare the dependence of both parameters on isoproterenol concentration (Fig. 4). Again the ability of isoproterenol to decrease the potential for contraction was used as an index for its effects on contractility. Prior incubation with isoproterenol caused concentration-dependent inhibition of contractions produced by subsequent exposure to carbachol. A 0.1 μM concentration of isoproterenol produced just insignificant inhibition ($p > 0.06$) of contraction; at 1 μM and greater, the drug elicited maximal responses. A similar dose dependence of the stimulation of cyclic AMP levels by isoproterenol was noted; i.e., at a concentration of 0.01 μM, where no effect on contractility was noted, isoproterenol also failed to alter cyclic AMP levels; at 0.1 μM a just significant increase in cyclic AMP was observed; and at 1 μM or greater, nearly maximal changes were noted. The concentration of isoproterenol required for half-maximal (ED_{50}) inhibition of contraction was 0.42 μM (0.22–0.80 μM; 95% confidence limits). The ED_{50} for the increase in cyclic AMP was almost identical: 0.41 μM (0.22–0.76 μM; 95% confidence limits).

Since smooth muscle cells in suspension were already relaxed, the inhibitory effect of isoproterenol was only measurable when cells were challenged with a test dose of carbachol. In order to determine whether the $t_{1/2}$ and ED_{50} values measured in the presence of carbachol really reflected the inherent properties of the reaction of isoproterenol with the cells rather than some interplay between the carbachol and isoproterenol effects, studies were performed to determine whether similar $t_{1/2}$ and ED_{50} values would be obtained if decreases in contractile force produced by isoproterenol could be determined more directly (i.e., in the absence of a contractile stimulus). To this end we compared the $t_{1/2}$ and ED_{50} of isoproterenol using 55 and 5.5 μM carbachol as a contractile stimulus. Although the contractile response was greater when cells were challenged with higher carbachol concentrations (the ratio of contractile responses was 1.53), the $t_{1/2}$ and ED_{50} for isoproterenol were identical (Table 2). It seems reasonable, therefore, to extrapolate from these data to the conditions under which the effects of isoproterenol on contractile force were determined in the absence of a contractile stimulus and conclude that similar $t_{1/2}$ and ED_{50} values would be obtained.

The ability of isoproterenol to inhibit

TABLE 1

Concentration of cyclic AMP in isolated smooth muscle cells

All values for each of these two experiments represent the means calculated for at least two 5-ml aliquots of a single cell suspension. Extracellular and intracellular values were determined by centrifugation of cells through a silicone oil layer (see METHODS).

Cyclic AMP content	Extracellular fluid volume	Intracellular fluid volume	Cyclic AMP concentration
pmoles/5 ml	*μl/5 ml*		*μM*
20.0	4.46	4.23	4.73
13.3	3.01	3.31	4.03

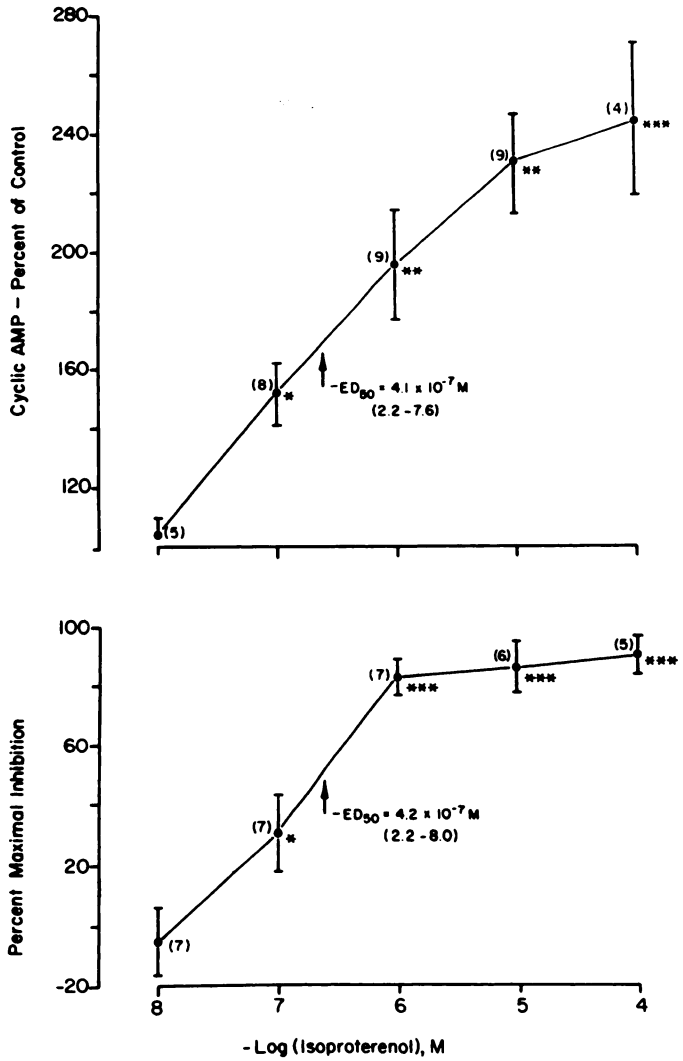

FIG. 4. *Dose-response curves for effects of isoproterenol on cyclic AMP levels (upper) and contractility (lower)*

Changes in cyclic AMP levels were determined at least 15 sec after the addition of isoproterenol. In each experiment changes in cyclic AMP levels were expressed relative to the levels in unstimulated cells. Each point represents the mean ± standard error calculated for data obtained from the number of experiments indicated in parentheses. The effects of isoproterenol on contractility were assessed indirectly as inhibition of carbachol-induced contractions (5.5 μM for 6 sec) after exposure to isoproterenol for at least 15 sec. Maximum inhibition of the response to carbachol was always observed at isoproterenol concentrations of 1 μM or greater. The extent of inhibition achieved at other concentrations of isoproterenol was expressed relative to the maximum achieved in each experiment. ED_{50} values and associated 95% confidence limits were calculated according to Spearman and Klaiber (17).

contractions was not confined to stimulation of the cells with carbachol. The magnitude of contractions elicited by exposing cell suspensions to 40 mM K^+ was also decreased by prior exposure to isoproterenol.

Although the above studies provide strong circumstantial evidence favoring the hypothesis that catecholamines may decrease contractility in smooth muscle by increasing cyclic AMP levels, more direct evidence was sought by examining

whether cyclic AMP and its dibutyryl derivative would also decrease the potential for contraction. Exposure of cell suspensions to dibutyryl cyclic AMP at concentrations as low as 100 μM caused significant inhibition of the carbachol responses (Table 3). Indeed, prior incubation with cyclic AMP also diminished contractions in response to carbachol, although 10-fold higher levels were necessary. Theophylline, thought to raise intracellular levels of cyclic AMP by inhibiting its breakdown (19), also attenuated carbachol-induced contraction (Table 3). In related studies, adenosine but not 5'-AMP was also found to decrease the potential for contraction. However, in contrast to the levels needed to produce changes in contractility in intact smooth muscles (20), relatively high concentrations (0.1–1 mM) of the nucleoside were necessary.

DISCUSSION

These studies demonstrate that isoproterenol can produce changes in both contractility and concentration of cyclic AMP in suspensions of isolated smooth muscle cells. The data are consistent with the hypothesis that an increase in cyclic AMP may be responsible for the decreased contractility in smooth muscle stimulated with *beta* adrenergic agents. In support of this contention, it was found that (a) isoproterenol caused an increase in cyclic AMP levels prior to a demonstrable decrease in contractility; (b) maximal changes in cyclic AMP levels were achieved at least 10 sec prior to maximal changes in contractility; (c) the ED_{50} values for the change in cyclic AMP and contractility were virtually identical; and (d) changes in both parameters occurred over a similar narrow range of isoproterenol concentrations. Furthermore, the observation that theophylline, cyclic AMP, and its dibutyryl derivative produced changes identical with those of isoproterenol argues that increases in cyclic AMP levels can lead to a decrease in contractility. Taken together, these data argue strongly that increases in cyclic AMP are causally related to the decreased contractility produced by *beta* adrenergic stimulation.

The present results are in agreement with an increasing number of studies with smooth muscle tissues, demonstrating a

TABLE 2

Effect of carbachol concentration on $t_{1/2}$ and ED_{50} values for isoproterenol

$t_{1/2}$ values represent the means ± standard errors for data obtained from three paired experiments at the two concentrations of carbachol. Cells were exposed to isoproterenol at a concentration of 10 μM. ED_{50} values were calculated according to Spearman and Klaiber (17). Limits are indicated in parentheses. Data were obtained from six paired experiments at the two concentrations of carbachol. Cells were exposed to isoproterenol (0.01–10 μM) for 30 sec prior to challenge with carbachol.

Carbachol stimulus (6 sec)	$t_{1/2}$	ED_{50}
μM	*sec*	μM
5.5	10.9 ± 1.5	0.33 (0.195–0.560)
55	10.5 ± 0.7	0.27 (0.174–0.417)

TABLE 3

Effects of cyclic AMP, dibutyryl cyclic AMP, theophylline, and adenosine on carbachol-induced contractions

Contractions were produced by a 6-sec exposure to carbachol at a concentration of 5.5 or 55 μM. In each experiment the response to carbachol following preliminary incubation with the agent indicated below is expressed relative to the response of aliquots exposed to carbachol without preliminary incubation (control). The negative response to carbachol seen after exposure to the higher concentration of dibutyryl cyclic AMP reflects instances when there was a decreased percentage of larger-amplitude pulses relative to unstimulated cells. Values are the means ± standard errors for the number of experiments indicated in parentheses.

Agent	Concentration	Exposure time	Response to carbachol
	mM	*min*	*% control*
None			100
Cyclic AMP	1	1	70 ± 12 (4)
Dibutyryl cyclic AMP	0.1	1	44 ± 12 (4)
	0.5	1	−2 (2)
Theophylline	1	2	56 ± 15.5 (5)
Adenosine	0.1	1	91 ± 9 (4)
	0.5	1	93 (2)
	1	1	43 ± 14 (5)

positive correlation between increases in cyclic AMP and a decrease in smooth muscle contractility produced by catecholamines (1–6). However, a number of other studies have provided evidence against this notion (8–10). Nesheim *et al.* (10) demonstrated that low doses of isoproterenol caused a decrease in the amplitude of spontaneous uterine contraction without leading to a measurable change in cyclic AMP level. Polacek and Daniel (9) demonstrated that whereas isoproterenol caused an increase in uterine cyclic AMP levels and a decrease in uterine motility, the subsequent addition of propranolol resulted in the resumption of spontaneous contractions although an increase rather than a decrease in cyclic AMP was observed. In a detailed study, Birnbaum *et al.* (11) found that the dose-response curve for isoproterenol-induced inhibition of uterine contractions was more than 2 log units to the left of the curve for the increases in cyclic AMP levels. However, since similar enantiomeric potency differences were noted for changes in cyclic AMP and mechanical activity, these authors argued against discarding the hypothesis that an increase in cyclic AMP mediates the relaxation. Instead they suggested that the heterogeneity of cell types present in the intact tissue may have obscured increases in cyclic AMP in relevant cells. It may well be that the data obtained by Nesheim *et al.* (10) and Polacek and Daniel (9) also reflect difficulties inherent in working with intact tissues.

The data in the present report provide evidence in favor of the hypothesis that *beta* adrenergic stimulation increases cyclic AMP and thereby brings about a decrease in contractility. One must be careful in extending this specific hypothesis to the more general one that an increase in cyclic AMP is the final mediator for all conditions that lead to a decrease in contractility. If this were so, a decrease in contractility would always be associated with an increase in cyclic AMP, and increases in cyclic AMP would always be associated with relaxation. This is not the case. Under certain conditions an increase in cyclic AMP has been associated with an increase in contractility (i.e., contractions) (8). Furthermore, under other conditions contractility has declined but without an associated increase in cyclic AMP (8, 21, 22). While these data argue against the general hypothesis that all decreases in contractility are mediated by an increase in cyclic AMP, the complexities inherent in intact smooth muscle make it difficult to draw this conclusion from negative evidence. For example, Murad and Kimura (23) demonstrated that prostaglandins and acetylcholine caused contraction of guinea pig tracheal muscle while also increasing cyclic AMP levels. As the authors pointed out, the changes in cyclic AMP need not necessarily be in smooth muscle cells or reflect a direct action of the drugs. The heterogeneous cell population raises the possibility that the increase in cyclic AMP occurred in non-muscle cells or was due to secondary release of endogenous catecholamine stores. Diamond and Holmes (8) found that depolarization of uteri by KCl increased cyclic AMP levels and caused the muscle to contract. This study did not specifically examine the likely possibility that the KCl released endogenous catecholamines from nerve terminals and that an increase in cyclic AMP occurred a a result. However, subsequent studies by Kroeger and Naimark (24) have shown that *beta* adrenergic blocking agents can blunt the rise in cyclic AMP and enhance the contractions produced by KCl. Therefore the findings of Diamond and Holmes could indeed reflect changes in cyclic AMP secondary to the action of KCl on smooth muscle. Again the advantage of using single cells to investigate a question of this type becomes apparent. In our studies with isolated smooth muscle cells we have failed to find an increase in cyclic AMP associated with contractions initiated with carbachol (Table 4) or with elevated K^+. Furthermore, we have never observed conditions under which decreased contractility induced by isoproterenol was not accompanied by increases in cyclic AMP. Even in the presence of carbachol, isoproterenol produced an increase in cyclic AMP levels and decreased contractility (Table 4). However,

TABLE 4

Effect of carbachol on cyclic AMP levels in the presence and absence of isoproterenol

Each value represents the mean ± standard error calculated from three aliquots of cell suspension.

Drug addition	Cyclic AMP content
	pmoles/5 ml
None	38 ± 4
Carbachol (5.5 μM), 15 sec	43 ± 3.5
Isoproterenol (1 μM), 15 sec	60 ± 5
Carbachol + isoproterenol	67 ± 7

while the evidence obtained on the isolated cells does not conflict with the general hypothesis that increases in cyclic AMP are the final mediator for relaxation, neither do they justify this general hypothesis. It seems likely that an increase in cyclic AMP causes relaxation by triggering a sequence of biochemical reactions. Thus it is quite probable that certain agents cause relaxation by a direct effect on a reaction in the sequence subsequent to cyclic AMP.

The basal cellular concentration of cyclic AMP in these studies, assuming a uniform distribution of the nucleotide in the intracellular space, is approximately 5 μM. This value is somewhat higher than values calculated for cyclic AMP content (1.2–2.4 μM)[3] of slices of toad muscularis, from which the cells were isolated. No previous studies on smooth muscle strips have measured the nucleotide and intracellular space simultaneously; however, if one assumes an intracellular space of 0.40 ml/g, wet weight, values for cyclic AMP concentration comparable to that determined for toad muscularis are obtained: 1.0 μM in rat uterus (8), 2.5 μM in rat vas deferens (3), 2.5 μM in rat uterus (5), and 1.4 μM in guinea pig taenia coli (25).

The drug-induced changes in both contractility and cyclic AMP levels reported in this study occurred more quickly ($t_{1/2}$ = 3 sec for increase in cyclic AMP) than has usually been demonstrated in studies with intact tissues [$t_{1/2}$ = 30 sec in rat myometrium (26), 1 min in guinea pig trachealis (23), and 1 min in rat myometrium (5)]. The rapidity of the changes in the cell suspensions most probably reflects the fact that all cells are exposed to drug almost instantaneously whereas only the most superficial cells would be immediately exposed in studies with intact tissues. The rapidity of the response to isoproterenol is all the more remarkable since these experiments were performed at 21° whereas those previously reported for intact mammalian preparations were performed at 37°. The maximal changes in cyclic AMP content in these studies (80–100%) are similar in magnitude to those found in some studies of intact mammalian tissues [100–200% (3, 5, 27)], although other studies have reported considerably larger maximal changes [500–700% (9, 23)].

Previous studies on intact smooth muscle strips have demonstrated that theophylline and dibutyryl cyclic AMP can exert inhibitory effects on smooth muscle (28–30). Our finding that prior incubation with these agents and with cyclic AMP can diminish carbachol-induced contractions in isolated cells demonstrates that these compounds can act directly on smooth muscle cells and that their actions in tissue are not due solely to release of endogenous neurotransmitters. It is presumed that theophylline, dibutyryl cyclic AMP, and cyclic AMP decrease contractility by inhibiting the breakdown of cyclic AMP or by mimicking a rise in intracellular cyclic AMP. However, this interpretation should remain guarded, since adenosine was also found to relax smooth muscle cells. While it is unlikely that dibutyryl cyclic AMP and cyclic AMP were degraded to adenosine, the structural similarity between adenosine and cyclic AMP, dibutyryl cyclic AMP, and theophylline raises the possibility that they may interact with the adenosine "receptor." Preliminary studies have also shown that adenosine (1 mM) can increase cyclic AMP levels, suggesting that its inhibitory actions may, like those of isoproterenol, be mediated by an increase in cyclic AMP levels.

It is well known that in smooth muscle muscarinic agents are excitatory and *beta*

[3] The cyclic AMP content of slices is 0.5–1.0 nmole/g, wet weight; the amount of intracellular water is 0.43 ml/g, wet weight.

adrenergic agents are inhibitory, and that in the presence of both stimuli an integrated response is obtained (31). It is unclear whether the integration is achieved at the cellular level (all the cells are responsive to both agents) or at the tissue level as a result of cell-to-cell coupling (cells have receptors for one agent, but not both). It is impossible to discern between these two possibilities with studies on intact tissues. However, since the isolated cells are no longer coupled, the results of the Coulter counter analysis indicate that individual smooth muscle cells contain receptors for both agents and therefore integration can occur at the cellular level. This conclusion is based on the following reasoning and observations: (a) the contraction induced by carbachol was entirely inhibited by isoproterenol; (b) the decrease in the proportion of contracted cells induced by isoproterenol might be due to its ability to relax either those cells normally contracted by carbachol or a different subpopulation of cells that are already contracted in the starting population; (c) the latter cannot be the case, since isoproterenol alone did not shift the distribution toward fewer contracted cells; (d) thus the ability of isoproterenol to inhibit completely the contractile effect of carbachol must be interpreted as evidence that the two agents act on the same cells. Since individual cells contain receptors for both muscarinic and *beta* adrenergic agents, the response of intact tissues to these two stimuli may be accounted for by integration at the level of the individual cells.

In summary, these studies have demonstrated the feasibility of investigating the biochemistry and physiology of smooth muscle freed of the complexities that have encumbered previous studies in intact tissues. The advantages of using isolated cells rather than intact tissues have been discussed. Further insight into the biochemical events associated with the contractile effects of isoproterenol, as well as other agents, ought to be facilitated by subsequent studies on smooth muscle cells in suspension.

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