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Potentiation of Isoproterenol-Induced Relaxation of Isolated Trachea by Aminophylline: Modulation by Desensitization (42558)

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Abstract. Continued exposure of many β-adrenoceptor-coupled adenylate cyclase systems to high doses of agonist causes diminished responsiveness, a phenomenon called desensitization. After exposure of isolated guinea pig tracheae to a high concentration of isoproterenol for 30 min, relaxation produced by subsequent challenge by a lower concentration was attenuated, as expected. However, potentiation of isoproterenol-induced relaxation by aminophylline was greater after desensitization as compared to that prior to desensitization. This observation was further investigated using a graphical method that allows quantitative and statistical evaluation of combinations of synergistically acting drugs. Concentration–relaxation curves (CRC) for isoproterenol alone and in the presence of a fixed concentration of aminophylline were determined in isolated rat trachea. A theoretical additive curve was constructed from the data obtained, and the displacement of the isoproterenol CRC from the theoretical additive curve caused by aminophylline in tracheae desensitized by 2.5 hr of exposure to 2×10^{-5} *M* isoproterenol (DESN) was compared to that in tracheae equilibrated for a similar period in physiologic salt solution (CON). Desensitization had no significant effect on aminophylline-induced relaxation but caused a marked depression and right-shift of the isoproterenol CRC. In the CON group aminophylline shifted the isoproterenol CRC upward and to the left indicating that the synergistic interaction between the two agents was greater than additive. The left-shift and elevation of the ceiling effect of the isoproterenol CRC caused by aminophylline were significantly greater in the DESN group vs the CON group. These observations from intact tissue are compared with published data from biochemical and broken cell studies. The possibility of increased phosphodiesterase activity as an explanation for the observations reported is discussed.

Based on data gathered from isolated cells (e.g., erythrocytes, cell culture) by ligand binding and biochemical techniques, several laboratories have constructed detailed hypotheses to explain stimulation of adenosine 3′:5′-cyclic monophosphate (cAMP) synthesis by β-adrenoceptor agonists (1–3). The resultant cAMP then mediates ensuing biochemical events characteristic of the given cell. Significantly, inactivation by phosphodiesterase also modulates the intracellular level of cAMP.

In most tissues and cells continued exposure of the β-adrenoceptor-coupled adenylate cyclase system to relatively high doses of agonist induces a decrease in responsiveness, a phenomenon called desensitization. Desensitization may take two forms, "homologous" which is specific for the desensitizing agonist and "heterologous" in which one agonist attenuates the response to other agonists acting through adenylate cyclase (4, 5). Evidence from biochemical experiments suggests that desensitization occurs in multiple steps, the kinetics of which vary widely among different cell types. As a result of the sequential nature of the process, the extent of involvement varies with time of exposure. Homologous desensitization may involve rapid uncoupling of β-adrenoceptors from other components of the adenylate cyclase system, sequestration of receptors from the cell surface, and actual loss of receptors. Heterologous desensitization may occur in cells in which homologous desensitization occurs but is usually slower in onset and may involve, among other factors, an increase in cAMP degradation (4–7).

While biochemical techniques employing individual cells and broken cell preparations have helped delineate specific steps in the complex chain of biochemical events mediating β-adrenoceptor-coupled adenylate cyclase responses, the experimental conditions required by biochemical techniques may distort the relationships existing between the various components in intact cells (3, 8). For this reason, these findings must be reconciled with observations in intact tissues.

This report describes observations made in

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isolated smooth muscle tracheal preparations. The observations are compared with published data obtained from biochemical experiments.

Materials and Methods. *Guinea pig experiments.* Adult male Hartley albino guinea pigs were stunned by a blow on the head, and the tracheae were removed with minimal trauma and cleaned of extraneous tissue. Stainless steel clips were placed in the lumen of approximately 12-mm segments of the tracheae; one clip was secured to the bottom of an isolated chamber bath (20 ml) and the other was connected by a silk thread to a Narco isometric force transducer. Changes in isometric tension were recorded on a Narco polygraph.

The tissues were bathed in physiologic salt solution (PSS) of the following composition [m*M*]: NaCl [118], KCl [4.7], KH_2PO_4 [1.2], $MgSO_4 \cdot 7HOH$ [1.2], $CaCl_2 \cdot 2HOH$ [2.5], NaHCO [25], glucose [11], EDTA·2Na·2HOH [0.02]. Temperature was maintained at 37°C throughout the experiment. The baths were bubbled with a gas mixture containing 5% carbon dioxide in oxygen.

The tracheae were equilibrated for 1 hr at 4 to 6 g of tension. A submaximal concentration (2.5×10^{-7} *M*) of carbachol was used to induce a state of contraction against which to measure relaxation caused by isoproterenol and aminophylline. When contractile tension had stabilized, isoproterenol (5×10^{-9} *M*) was added to the bath and relaxation was allowed to reach completion. The baths were drained, fresh PSS was added, and the tissues were allowed to return to the initial baseline. They were again contracted with carbachol and when contraction had stabilized, aminophylline (1.4×10^{-5} *M*) was added to the bath. After the relaxation had stabilized and was recorded, isoproterenol (5×10^{-9} *M*) was added to the bath and the combined response was recorded.

The tissues were then exposed to a desensitizing concentration (5×10^{-6} *M*) of isoproterenol for 30 min after which they were washed with PSS at 5-min intervals for 30 min. Responses to aminophylline and isoproterenol, alone and combined, were again obtained as described above, except that the tissues were reexposed to 5×10^{-6} *M* isoproterenol before the final administration of aminophylline and isoproterenol.

Relaxation was measured as the decrease in tension from the carbachol-induced contractile tone, and was expressed as a percentage of the contraction caused by carbachol.

Rat experiments. Female outbred albino rats (approximately 250 g) were stunned, and the tracheae were removed and prepared for measurement of isometric tension as described above. One group of tissues (Control) was equilibrated at 2 to 4 g of tension for 2.5 hr in PSS while another group (Desensitized) was exposed to 2×10^{-5} *M* isoproterenol. The solution in the bath was changed every 20 min during this equilibration period after which the tissues were washed with fresh PSS every 10 min for 30 min.

After equilibration the tissues were contracted with 5×10^{-5} *M* carbachol, and increasing concentrations of isoproterenol were added cumulatively to the bath. Maximum relaxation for each tissue was determined by addition of a supramaximal concentration of tetracaine (1×10^{-4} *M*). The rat trachea makes an ideal preparation for studying synergism of isoproterenol- and aminophylline-induced relaxation because the maximal relaxation produced by isoproterenol is less than that produced by tetracaine. This allows observation of any increase in the ceiling response to isoproterenol caused by aminophylline.

The baths were drained, and the tissues of the Control group were re-equilibrated in fresh PSS. Preliminary experiments suggested that desensitized trachea tended to regain sensitivity to isoproterenol after removal from the high-desensitizing concentration. Since determination of the first isoproterenol concentration–response curve required a significant period of time, the tissues of the Desensitized group were reexposed to 2×10^{-5} *M* isoproterenol. After 1 hr all the tissues were washed with fresh PSS every 10 min for 30 min and then again contracted with carbachol. Aminophylline (1.4×10^{-5} *M*) was added to the bath, relaxation was recorded, and a second isoproterenol concentration–relaxation curve was determined in the presence of aminophylline.

The data obtained from these experiments were analyzed by the method of Pöch and Holzmann (9). This graphical method allows quantitative and statistical evaluation of com-

binations of synergistically acting drugs. A theoretical additive concentration–response curve is constructed from the experimental concentration–response curve of one drug and the response to a fixed concentration of a second drug. This theoretical additive curve extends from the second drug's effect to the ceiling effect of the first drug. A second experimental concentration–response curve is determined in the presence of the second drug and compared with the theoretical additive curve. An additive drug interaction is characterized by coincidence of the experimental and theoretical curves with respect to location on the concentration axis and magnitude of ceiling effect. An overadditive synergism is characterized by a shift to the left and an increased ceiling for the combined drugs as compared with the theoretical additive curve. The magnitude of the shift is measured by the ratio of ED_{50} doses for the two curves. This dose ratio or dose factor, as well as the increase of the ceiling effect, can be analyzed statistically allowing quantitative comparison of different drug combinations or, in the case of this report, the effect of experimental conditions on the synergistic interactions between two drugs.

Drugs. *dl*-isoproterenol · HCl, carbamylcholine chloride (carbachol) and aminophylline([theophylline]$_2$ethylenediamine) were obtained from Sigma Chemical Co. Working solutions were made in PSS; isoproterenol working solutions were kept on ice.

Analysis of data. Comparison of individual responses obtained in the same tissue was made by analysis of variance (ANOVA) for paired comparisons. Comparison of responses obtained in different tissues (i.e., Control vs Desensitized groups, rat experiments) was made by a simple one-way ANOVA. Student's *t* test was used to test for deviation of the log dose factor from zero, the theoretical value expected for additive synergism.

Results. *In vitro* exposure of isolated guinea pig trachea to 5×10^{-6} *M* isoproterenol for 30 min did not significantly affect relaxation caused by 1.4×10^{-5} *M* aminophylline (Fig. 1). In contrast, it markedly attenuated the response to 5×10^{-9} *M* isoproterenol. Although the relaxation caused by combined administration of both agents was reduced after desensitization as compared with that prior to desensitization, the magnitude of the decrease appeared less than would be expected given the marked attenuation of the isoproterenol response. Prior to desensitization, combined administration of isoproterenol and aminophylline caused a mean response 36% greater than the sum of the mean responses to the same doses of these agents when administered individually. After desensitization the mean response to combined administration was 126% greater than the sum of the individual mean responses.

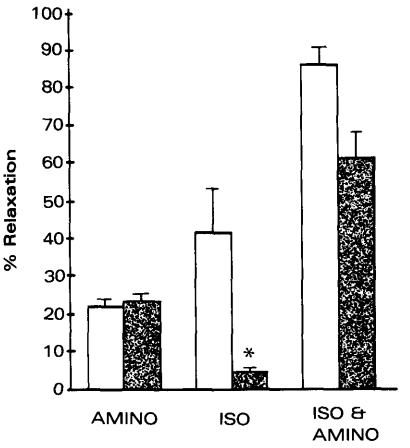

FIG. 1. Effect of acute desensitization on relaxation of guinea pig trachea caused by aminophylline (Amino) and isoproterenol (Iso), alone and combined. Relaxation of isolated guinea pig trachea was measured as the decrease in tension from carbachol-induced contraction and was expressed as percentage of the magnitude of contraction caused by carbachol. The bars represent the mean ($n = 4$) relaxation caused by Amino (1.4×10^{-5} *M*) and Iso (5.0×10^{-9} *M*) administered alone and in combination, prior to (open bars) and after (stippled bars) exposure to 5×10^{-6} *M* Iso for 30 min. Standard errors of the mean are represented by the vertical lines. *Significantly different ($P < 0.05$) from the response prior to desensitization.

The above observations, while suggestive, are limited by the inherent danger of comparing the effect of combined administration of two drugs to the sum of their individual actions as pointed out by Pöch and Holzmann (9). For this reason, a series of experiments was performed in rat trachea following their method which allowed determination of the entire isoproterenol concentration–response curve alone and in the presence of a fixed con-

centration of aminophylline (see Materials and Methods). Similar to the results observed in guinea pig trachea, exposure to 2×10^{-5} *M* isoproterenol for 2.25 hr had no statistically significant effect on the relaxation caused by aminophylline but caused a marked right-shift and depression of the isoproterenol concentration–relaxation curve (Fig. 2).

Figure 3 illustrates the concentration–relaxation curves for isoproterenol alone and in the presence of 1.25×10^{-4} *M* aminophylline. The dotted line represents the theoretical additive curve calculated from the responses to aminophylline (vertical bar) and isoproterenol alone. The horizontal line illustrates the log dose factor (LDF) derived from the theoretical additive and experimental combined concentration–relaxation curves. The mean LDF was tested by Student's *t* test against the null hypothesis of LDF = 0 as would be expected for additive synergism and found to be significantly different ($P < 0.001$; 6 *df*). The geometric mean and 95% confidence limits were 3.4 (2.2, 5.3). The maxima estimated from the individual concentration–relaxation curves were tested by ANOVA for paired comparisons and the maxima of the isoproterenol curves in the presence of aminophylline were significantly different ($P < 0.01$) from those of

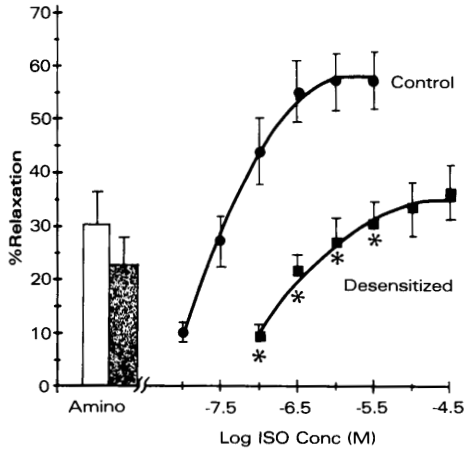

FIG. 2. Effect of acute desensitization on aminophylline- and isoproterenol-induced relaxation of rat trachea. One group (Desensitized; $n = 8$) of isolated rat tracheae was exposed to 2×10^{-5} *M* isoproterenol (Iso) for 2.25 hr while the control group (Control; $n = 7$) was equilibrated in normal physiologic salt solution. After contraction with 5×10^{-6} *M* carbachol, aminophylline (Amino; 1.25×10^{-5} *M*) or increasing concentrations of Iso were added to the bath. After relaxation had stabilized, a maximally effective concentration of tetracaine (1×10^{-4} *M*) was added. Relaxation was measured as the change in tension from the carbachol-induced contractile tone and expressed as percentage of the response to tetracaine. Amino-induced relaxation in the Desensitized group (stippled bar) was not significantly different from that of the Control group (open bar). Iso-induced relaxation in the Desensitized group at the doses indicated (*) were significantly ($P < 0.01$) different from the respective values of the Control group. The bars and points represent mean relaxation; the vertical lines represent the standard errors of the mean.

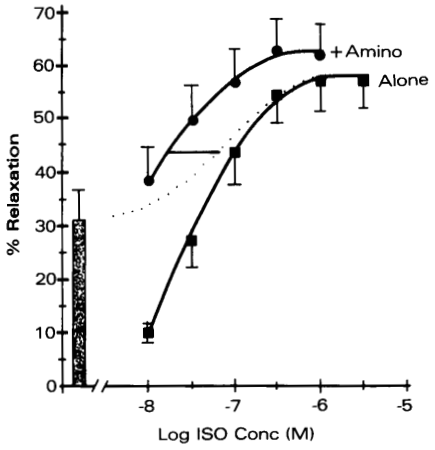

FIG. 3. Synergism between isoproterenol (Iso) and aminophylline (Amino) in naive rat trachea. Isolated rat tracheae ($n = 7$) were contracted with 5×10^{-6} *M* carbachol and increasing concentrations of Iso were added in a cumulative manner (Alone). After the response to the last concentration of Iso had stabilized, a maximally effective concentration of tetracaine (1×10^{-4} *M*) was added to the bath. Following a reequilibration period, the tissues were again contracted with carbachol and 1.25×10^{-5} *M* Amino was added to the bath. After the response (stippled bar) had stabilized, a second Iso cumulative concentration–response curve was determined (+ Amino). Relaxation was measured as the change in tension from the carbachol-induced contractile tone and expressed as percentage of the response to tetracaine. The responses indicated represent the means and standard errors of the means. The dotted line represents the theoretical curve expected if the response to combined administration to Iso and Amino was due to additive synergism (see Material and Methods). The horizontal line connecting the theoretical curve for additive synergism and the Iso concentration–response curve in the presence of Amino illustrates the log dose factor by which the experimental curve deviates from the theoretical.

isoproterenol alone. The means ± SEM were 61.2 ± 6.2% and 56.4 ± 5.7%, respectively.

Figure 4 resembles Fig. 3 except that the tissues were exposed to 2×10^{-5} *M* isoproterenol prior to determination of the concentration–relaxation curves. The mean LDF was significantly different from zero ($P < 0.01$; 7 *df*); the geometric mean and 95% confidence limits were 30.0 (3.7, 242.5). The maxima estimated from the individual isoproterenol concentration–relaxation curves in the presence of aminophylline were significantly different ($P < 0.01$) from those of isoproterenol alone. The respective means ± SEM were 47.9 ± 4.3% and 35.9 ± 4.6%.

The data given in Table I illustrate aminophylline's potentiation of isoproterenol-induced relaxation. Desensitization was associated with a significantly greater ($P < 0.05$) left-shift of the isoproterenol concentration–relaxation curve by aminophylline. In addition, aminophylline caused a significantly greater ($P < 0.05$) elevation of isoproterenol's ceiling effect in the desensitized group as compared with the control group.

Discussion. Aminophylline increased isoproterenol-induced relaxation in both untreated guinea pig and rat trachea. The significant left-shift and elevation of the ceiling effect of the isoproterenol concentration–relaxation curve (Fig. 3) from that predicted by additive synergism suggest that aminophylline's effect on the relaxation produced by isoproterenol was not one of simple additive synergism but one of potentiation. This finding is consistent with the hypothesis that β-adrenoceptor-mediated tracheal relaxation results from activation of adenylate cyclase and increased intracellular levels of cAMP while aminophylline inhibits phosphodiesterase inactivation of intracellular cAMP (1, 4, 10–12).

Exposure of both guinea pig and rat tracheae to high concentrations of isoproterenol markedly attenuated the relaxation caused by isoproterenol but had no statistically significant effect on aminophylline-induced relaxation (Figs. 1 and 2). These observations suggests a possible decrease in isoproterenol-activated cAMP synthesis with no change in basal cAMP turnover. However, if decreased isoproterenol-induced cAMP synthesis alone was responsible for the attenuated isoproterenol-induced relaxation observed, one would expect desensitization to have attenuated proportionately the response to isoproterenol in the presence of aminophylline. This was not the case (Fig. 1; c.g. Figs. 3 and 4; Table I).

The marked increase in aminophylline's

TABLE I. EFFECT OF DESENSITIZATION ON THE POTENTIATION OF ISOPROTERENOL-INDUCED TRACHEAL RELAXATION BY AMINOPHYLLINE

	Log dose factor[a]	Difference[b] in maxima
Control ($n = 7$)	0.54 ± 0.08	4.9 ± 1.2
Desensitized ($n = 8$)	1.48 ± 0.38*	12.0 ± 2.9*

Note. Data are the means and SEM calculated from the individual concentration-relaxation curves represented by Figs. 3 and 4.

[a] Shift in the isoproterenol concentration–relaxation curve caused by aminophylline from the theoretical curve predicted by additive synergism as measured by the difference between the respective log ED_{50} values.

[b] Difference in the ceiling response to isoproterenol alone and in the presence of aminophylline (percentage of tetracaine maximum).

* Significantly different from control value ($P < 0.05$); ANOVA of individual values.

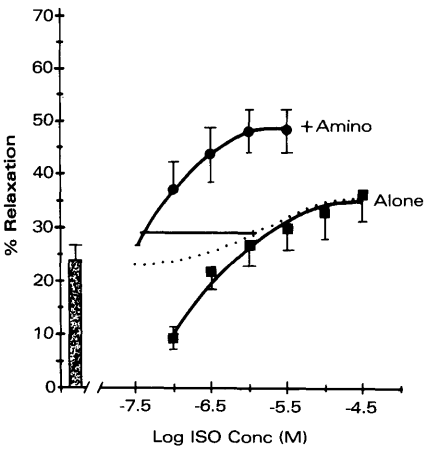

FIG. 4. Effect of acute desensitization on synergism between isoproterenol (Iso) and aminophylline (Amino) in rat trachea. The data represented were obtained as described in Fig. 3 except that during the initial equilibration period and the reequilibration period between determination of the concentration curves, the tracheae ($n = 8$) were exposed to 2×10^{-5} *M* isoproterenol (Iso) for 2.25 and 1 hr respectively.

potentiation of isoproterenol-induced relaxation after desensitization suggests that other factors, in addition to decreased synthesis, may have contributed to the desensitization observed. Similar observations have been reported in cultured cell preparations. For example, Fishman *et al.* (13, Table 6) reported that incubation of cultured rat glioma cells with 10 μM isoproterenol for 3 hr markedly decreased cAMP accumulation resulting from subsequent challenge with isoproterenol but basal cAMP accumulation was unaffected. Similar to the observations of the present experiments employing intact tissue, addition of a methylxanthine phosphodiesterase inhibitor caused an almost sixfold increase (corrected for basal accumulation) in isoproterenol-induced cAMP accumulation in desensitized C6LP cells as opposed to a twofold increase in naive cells. Similar data were reported by Browning *et al.* (14, Table 1).

Desensitization of the β-adrenoceptor-coupled adenylate cyclase system appears to involve several steps with different kinetic characteristics. In most *in vitro* systems a rapid uncoupling of the β-adrenoceptor from adenylate cyclase appears to occur followed by a loss of high-affinity agonist binding (4, 6, 7). Continued exposure to the agonist results in a loss of β-adrenoceptors.

In addition to the above changes related to the β-adrenoceptor and its coupling to the adenylate cyclase enzyme, some *in vitro* studies have reported an increase in the rate of cAMP hydrolysis by phosphodiesterase in cells exposed to high concentrations of adrenergic agonists. Browning *et al.* (14) found that C6 astrocytoma cells incubated for 3 hr with norepinephrine lost the ability to respond to norepinephrine by accumulating high concentrations of cAMP as compared with naive cells. Their data indicated that the attenuated accumulation of cAMP by the treated cells resulted from a more rapid hydrolysis of cAMP due to increased cyclic nucleotide phosphodiesterase activity. Bourne *et al.* (15), in mouse lymphoma cells, and Schartz *et al.* (16), in C6 rat glioma cells, reported that increased cAMP, whether endogenous as a result of exposure to catecholamines or as exogenously applied dibutyryl cAMP, was associated with increased phosphodiesterase activity.

The participation of increased degradation of cAMP in the desensitization phenomenon appears to depend on the dose and time of exposure to the desensitizing agent. Su *et al.* (17) found that the rate constant for cAMP degradation in human astrocytoma cells (1321N1) exposed to catecholamines and PGE_1 did not change during the first 60 min of exposure even though cAMP accumulation was diminished. They concluded from this observation that increased degradation was not involved in agonist-specific desensitization occurring within the first 60 min of exposure. They also noted, however, that the rate constant increased significantly after 90 min and was increased twofold after 180 min of exposure.

At least two forms of phosphodiesterase, one with high and one with low affinity for cAMP, have been shown to exist in various tissues (12, 18, 19). Uzunov *et al.* (20) studied the effects of a protein activator extracted from rat brain on highly purified low-affinity phosphodiesterase from both rat brain and frog sympathetic chain. They found that the activator decreased the K_M of this enzyme for cAMP fourfold bringing the affinity of the enzyme into the range of intracellular levels, especially after stimulation of synthesis by hormones and neurotransmitters. A similar increase in activity of a low-affinity form of phosphodiesterase caused by desensitization would be consistent with the data observed in the present study since desensitization did not affect the relaxant action of aminophylline itself but significantly potentiated the effects of isoproterenol.

Although the reports cited above suggest a possible role for inhibition of phosphodiesterase by aminophylline in explaining the observations reported in the present study, it should be noted that intact tissue was employed and phosphodiesterase activity was not measured. Any discussion of changes in its activity, therefore, must remain speculative. In addition, the doses of aminophylline used were lower than those now thought to produce significant phosphodiesterase inhibition in most tissues (21). The methylxanthines produce other actions, i.e., modulation of cellular calcium mobilization and inhibition of adenosine receptors, whose involvement in the ob-

servations reported in this study cannot be ruled out.

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