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Effect of trimetoquinol on the cyclic AMP level in the tracheal muscle of guinea-pig

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THE RELAXATION of smooth muscle induced by adrenergic β-stimulating agents is associated with an elevation of intracellular levels of cyclic AMP (c-AMP) in guinea-pig[1] and rabbit[2] intestine and rat[3] uterus, although there are some contrary observations.[4] Using tracheal ring preparations of guinea-pig, Murad demonstrated that epinephrine caused an increase of c-AMP in the tissue.[5] Correlation of the cyclic nucleotide level to the relaxation was also studied by Moore *et al.* using tracheal chain preparations of guinea-pig[6] and by Triner *et al.* using blood vessels of various mammalian species.[7] However, the tracheal ring consists mostly of pharmacologically inert connective tissue that must be removed for exact determination of the intracellular level of c-AMP in the muscular tissue which occupies only a small portion of the ring. A study described in this paper was aimed to determine unequivocally whether c-AMP levels increase in tracheal muscle of guinea pig in response to a new β-adrenergic bronchodilator, trimetoquinol (*l*-1-(3,4,5-trimethoxybenzyl)-6, 7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrochloride, TMQ)[8] and also to examine whether the increase in c-AMP level occurs prior to or in parallel with the pharmacological response of the tissue to the drug.

Immediately after decapitation of male guinea pigs (250–350 g body wt), tracheas were excised and placed in a cold Tyrode's solution containing 0·1% (w/v) glucose and saturated with a mixed gas of 95% O_2 and 5% CO_2. The medium was continuously aerated with the same gas mixture during all experiments. After removing the adipose tissue attached on the outer surface of the trachea, the muscular tissue was carefully cut out longitudinally free of the connective tissue. Some specimens of the muscle were examined histologically. Each muscle strip was preincubated in the above medium (20 ml) for 20 min at 37° and then transfered to fresh medium (20 ml). Incubation was started by addition of the test compound, 10^{-7} M TMQ or isoproterenol (Iso). In the inhibition experiments, propranolol (10^{-5} M) was added to the medium 2 min prior to the addition of TMQ. Incubation was terminated by dipping the strip into 1 ml of hot distilled water in a test tube in a boiling water bath for 3 min. After cooling, the contents of the test tube were homogenized with a glass homogenizer. After centrifugation (1500 *g*, 20 min), the supernatant fraction was used for determination of c-AMP by the protein binding method[9,10] and the precipitate for protein determination by Lowry's method.[11] c-AMP phosphodiesterase (PDE) of tracheal muscle homogenates was assayed according to the method of Brooker *et al.*[12] modified by Thompson and Appleman.[13] The homogenates for PDE assay were prepared by homogenizing tracheal muscle in 20 vol of 0·33 M sucrose with a glass homogenizer for a total of 5 min with two 1-min intermissions in ice water. The PDE reaction was started by adding the properly diluted homogenate to the incubation mixture containing 0·4 μCi of 8-[^{3}H]-c-AMP (RCC, Amersham) in a final volume of 0·4 ml. After 15-min incubation at 37°, the reaction was terminated by boiling for 2·5 min. 5'-nucleotidase reaction with 10 μg (0·1 ml) of Crotalus adamanteus venom (BDH Chemicals Ltd, Poole) for 10 min at 37° was stopped by adding 1 ml of a 50% aqueous suspension of anion-exchange resin (Bio-Rad AG 1-X2, 200–400 mesh). After centrifugation, an aliquot of supernatant was counted in the presence of scintillation fluid consisting of 50 g of naphthalene, 5 g of 2,5-diphenyloxazole, 0·3 g of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene, 730 ml of *p*-dioxane, 135 ml of toluene and 35 ml of methanol. It was confirmed that the drugs tested did not affect the 5'-nucleotidase activity.

For the relaxation experiments, isolated tracheal chains of guinea-pigs (250–350 g) were prepared according to the method of Takagi *et al.*[14] and suspended in an organ bath containing Tyrode solution (20 ml) which was kept at 36° ± 1° and continuously bubbled with air. Relaxation responses produced by TMQ (10^{-7} M) were magnified approximately 18 times by means of a writing lever (about 150 mg) and recorded on a kymograph isotonically.

Our preliminary experiments with tracheal ring preparations of guinea-pig failed to give conclusive results, because the ring preparations, even after the muscle tissue had been removed, showed a moderate (about 50 per cent) increase in the c-AMP level in response to TMQ (from the basal level of about 2 pmoles/mg protein to about 3 pmoles/mg protein). Murad[5] also mentions the presence of heterogenous cell types in the tracheal ring which might contribute to the overall increase of c-AMP. In our tracheal muscle preparations, addition of TMQ in the incubation medium resulted in a 2-fold increase in the c-AMQ level within 1 min (Fig. 1b). Even at 20 sec after the start of incubation, a significant increase in the c-AMP level was observed. The elevated level of c-AMP at 1 min was sustained during further incuba-

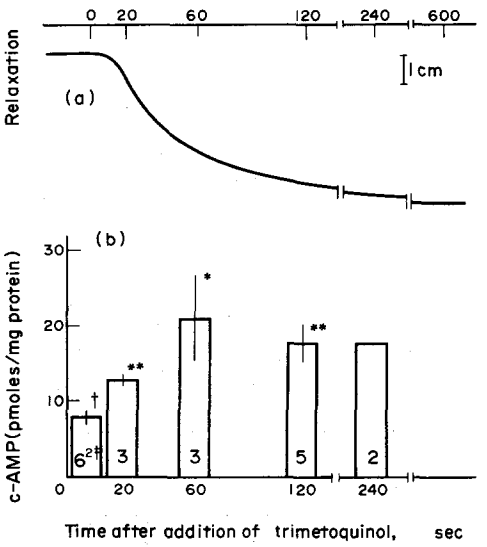

FIG. 1. Time–response relationships of the action of TMQ (10^{-7} M) on (a) isotonic relaxation of a tracheal chain and (b) c-AMP levels of the muscle strips. TMQ was added to the medium at zero time. †Mean ± S.E., ‡number of strips (animals), *P < 0·02, **P < 0·01 (compared with the value at zero time).

tion up to 4 min. In a separate experiment with a tracheal chain preparation, relaxation was observed only to the extent of about 20 per cent of the maximal magnitude at 20 sec after addition of TMQ (10^{-7} M). The relaxation was still much smaller than the maximum at 1 min when the level of intracellular c-AMP in the tracheal muscle strips had already reached the maximum. Several minutes or more were needed for the tracheal chain to relax to the maximal amplitude (Fig. 1a). Even if the baseline on the kymogram was lowered by about 30 per cent of the maximal amplitude, and consequently the magnitude of the relaxation was that much decreased, by putting a weight of about 250 mg at 1 cm away from the fulcrum on the lever, the time course of relaxation did not change. The significant increase of c-AMP during the short term incubation in the presence of the adrenergic β-receptor stimulant as shown in Fig. 1b is

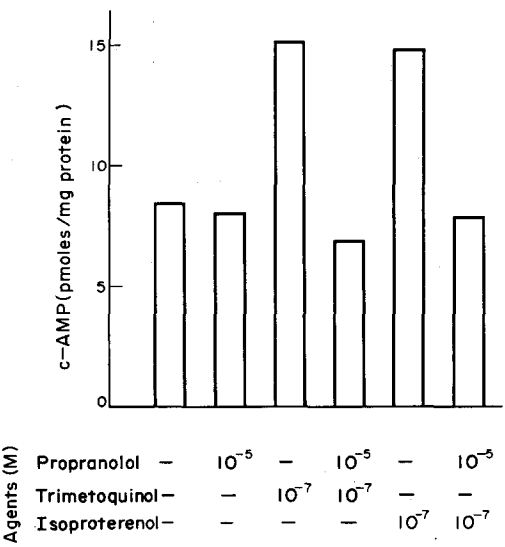

FIG. 2. Effect of propranolol on TMQ-induced increase of c-AMP in the muscle preparation. Tracheal muscle strips were incubated for 2 min after addition of TMQ or Iso at 37°. Propranolol was added 2 min prior to the addition of TMQ or Iso. Each value is the mean of three experiments.

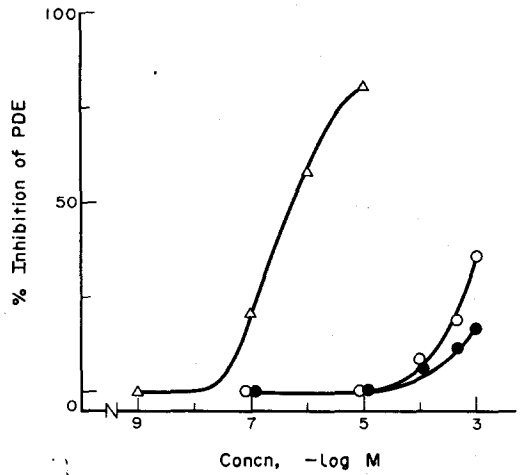

FIG. 3. Effects of smooth muscle relaxants on c-AMP phosphodiesterase activity of tracheal muscle homogenate. The reaction was carried out at 2×10^{-7} M of c-AMP. The basal activity was 5·6 pmoles/mg protein/min. (○) TMQ, (●) Iso, (△) papaverine.

in line with the results obtained by other investigators using guinea-pig taenia caecum,[1] rabbit colon[2] and rat uterus,[3] and suggests that an increase of intracellular c-AMP precedes the relaxation of smooth muscle induced by adrenergic β-stimulating drugs.

Histological examination of the muscle preparations used in the present study revealed that they mostly consisted of smooth muscle cells, and contained only a slight amount of connective tissue elements. Consequently, contribution of the amount of c-AMP in the connective tissue should be insignificant. Therefore, the increase in the c-AMP level of the muscle preparations induced by TMQ can be regarded as an increase in the c-AMP level in tracheal muscle cells.

The increase of c-AMP by TMQ was completely blocked in the presence of adrenergic β-blocking agent propranolol, indicating that the increase of c-AMP induced by TMQ was mediated by an adrenergic β-receptor (Fig. 2).

The effect of TMQ on c-AMP PDE was examined in order to exclude the possibility that TMQ might have increased c-AMP through inhibition of c-AMP PDE like the smooth muscle relaxant papaverine.[7,15,16] As shown in Fig. 3, low concentrations of TMQ and Iso which resulted in increases in the tissue c-AMP level did not affect c-AMP PDE activity in the homogenate of tracheal muscle at all, although papaverine markedly inhibited the enzyme activity at these concentrations. Therefore, the action of TMQ at lower concentrations on the tracheal muscle is likely to be a result of activation of the tissue adenylate cyclase. Concentrations higher than 10^{-5} M of TMQ and Iso brought about some inhibition of the c-AMP PDE activity. This inhibition at higher concentrations may correlate with the papaverine-like action of TMQ on depolarized smooth muscle.[8]

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An acute effect of high oxygen tension on the uptake of ^{3}H-deoxycytidine into thymocyte deoxyribonucleic acid

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IT IS GENERALLY believed that use of oxygen tensions of greater than 30 per cent in mammalian cell cultures will inhibit growth and nucleic acid synthesis.[1] Andersen *et al.*,[2] using human lymphocytes stimulated with phytohemagglutinin, showed that an atmosphere of 5–20% O_2 gave optimal rates of incorporation of ^{3}H-thymidine in 68-hr cultures. After 48 hr of culture employing 80% O_2, myocardial cells displayed depressed ^{14}C-uridine incorporation compared to cells grown under 20% O_2.[3] However, little data are available concerning the acute effects of high oxygen tension on nucleic acid metabolism in mammalian cells.

Thymic lymphocyte suspensions were prepared from 2 to 4 Carworth Farm rats that had been maintained on Rockland Lab Chow and tap water *ad lib.* for at least 1 week. The animals were sacrificed by decapitation, and the thymuses were placed in ice-cold minimal essential medium (MEM Joklik modified, Grand Island Co., New York) that had been gassed previously with 90% air–10% CO_2 or 90% O_2–10% CO_2 to bring the pH to 7·0–7·2. Cells were teased out of the thymus tissue, and the suspension was filtered through a 250 mesh stainless steel screen to remove the connective tissue. The thymocytes were washed three times at 0–4° with MEM using centrifugation at 600 *g* for 2 min. The cell suspension was made up to 3·0 ml and counted by standard hematological techniques.

Cultures of 1·5 ml containing 4·0 × 10^7 cells/ml were gassed, capped and preincubated in duplicate for 0–3 hr at 37°. At the appropriate time, 0·5 ml of each cell suspension was pulsed in duplicate under the same gas for 1 hr with 1 μCi of a ^{3}H-nucleoside in 0·1 ml MEM. Incorporation of the radioactivity was terminated by the rapid addition of ice-cold MEM containing 1 × 10^{-4} M non-radioactive nucleoside. The cells were centrifuged, the supernatant was aspirated, and the cell button washed with 5% trichloroacetic acid (TCA) on a nitro cellulose filter (Schleicher & Schuell, average pore size 0·45 μm). The filters were dried under a heat lamp and placed in 15 ml scintillation fluid[4] for at least 18 hr prior to counting in a Nuclear–Chicago Unilux II liquid scintillation counter. Quenching was determined to be essentially constant for all tubes; therefore, results are reported in cpm for the 2·0 × 10^7 cells counted. All glassware which came into contact with the cells during incubation or labeling was siliconized prior to use and washed in 7X nonionic detergent.

Radioisotopes were purchased from Schwarz Bioresearch, Inc. and had specific activities of 26·2, 3·0 and 2·0 Ci/m-mole for deoxycytidine-5-^{3}H, thymidine-methyl-^{3}H and uridine-5-^{3}H respectively.

Figure 1 shows the effect of high and low oxygen tensions on the relative rates of incorporation of ^{3}H-nucleosides into nucleic acids. Incorporation rates are compared to those obtained without preincubation. Using 90% O_2-10% CO_2 as the gas phase, uptake of ^{3}H-deoxycytidine into DNA declined rapidly over