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EFFECT OF GLUCAGON, DIBUTYRYL ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE AND PHOSPHODIESTERASE INHIBITORS ON RAT LIVER PHOSPHORYLASE ACTIVITY AND ADENOSINE 3′,5′-CYCLIC MONOPHOSPHATE LEVELS*

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Abstract—Theophylline, papaverine and 1-ethyl-4-(isopropylidenehydrazine)-1H-pyrazole-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester HCl (SQ 20,009) were found to inhibit both high K_m (200 μM) and low K_m (3 μM) cyclic 3′,5′-AMP phosphodiesterase activity in 100,000 *g* supernatant fraction from rat liver. Theophylline was the least potent and SQ 20,009 the most potent inhibitor of both activities. In the liver slice preparation, papaverine (5×10^{-4}), theophylline (10^{-3} M) and SQ 20,009 (10^{-3} M) failed to elevate cyclic 3′,5′-AMP, while glucagon (10^{-6} M) produced a significant ($P < 0.05$) elevation in the tissue levels of the cyclic nucleotide. All three phosphodiesterase inhibitors significantly ($P < 0.05$) augmented the ability of glucagon to elevate cyclic 3′,5′-AMP levels. Glucagon and papaverine stimulated phosphorylase activity, while theophylline inhibited both basal and glucagon-stimulated phosphorylase activity. SQ 20,009 was without effect. It is concluded that no cause and effect relationship can be assumed between an ability to inhibit phosphodiesterase and to alter phosphorylase activity.

SUTHERLAND, ROBISON *et al.*[1,2] have demonstrated that hormone activation of glycogenolysis involves activation of adenylate cyclase and increased formation of cyclic 3′,5′-AMP. Increased levels of cyclic 3′,5′-AMP presumably activate a protein kinase which together with ATP phosphorylates glycogen phosphorylase, converting it from an inactive to an active form.[3] Tissue levels of cyclic 3′,5′-AMP may also be increased by the inhibition of cyclic 3′,5′-AMP phosphodiesterase, the enzyme which metabolizes cyclic 3′,5′-AMP to 5′-AMP. The methylxanthines were the first group of compounds to be identified as inhibitors of this enzyme.[4] Subsequently, a number of drugs have been identified as phosphodiesterase inhibitors.[5–8] In the present study, the effects of three inhibitors, papaverine, 1-ethyl-4-(isopropylidenehydrazine)-1H-pyrazole-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester HCl (SQ 20,009) and theophylline, alone and in combination with glucagon, have been compared on activation of rat liver phosphorylase, phosphodiesterase activity and tissue 3′,5′-AMP levels.

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MATERIALS AND METHODS

Animals. Fasted male, Cox rats weighing between 180 and 200 g were used for all studies. Animals were sacrificed by a blow on the head and exsanguination. Livers were rapidly removed and chilled on ice prior to slicing with a Stadie Riggs hand microtome.

Metabolic studies. Rat liver slices (350–500 mg) were preincubated for 30 min in 5 ml of a Krebs–Ringer bicarbonate buffer, gassed with 95% O_2–5% CO_2. Slices were then transferred to a new medium containing glucagon and/or papaverine, theophylline or SQ 20,009. Tissues were incubated an additional 5–15 min.

Cyclic 3',5'-AMP levels. Cyclic 3',5'-AMP was assayed by a modification of the method of Gilman.[9] After incubation, tissues were removed from incubation flasks and homogenized in 3 ml of cold 5% trichloroacetic acid containing 0·1 ml of 1 N HCl. The samples were centrifuged for 5 min and the supernatant fluid was extracted three times with 5 vol. of water-saturated diethyl ether. The samples were lyophylized and reconstituted in 200 mM sodium acetate buffer, pH 4·0, to give a final concentration of 50 mg (wet wt of tissue/ml). The incubation mixture contained sufficient binding protein obtained from rabbit skeletal muscle to bind 30 per cent of the added cyclic 3',5'-AMP, inhibitor protein and ^{3}H-cyclic 3',5'-AMP (5 pmoles: 0·1 μCi). The reaction was initiated by the addition of binding protein and was incubated for 60 min at 0°. At equilibrium, the mixture was diluted to 1 ml with cold 20 mM sodium phosphate buffer, pH 6·0, and filtered through a Millipore filter (0·45 μm). The filter was washed with 10 ml of the same buffer and dissolved in scintillation fluid. The latter consisted of two parts toluene containing 5·32 g/l of 2·5-diphenyloxazole (PPO) and 266 mg of dimethyl 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) and one part Triton X-100.

Phosphorylase activity. Phosphorylase activity was assayed as previously described.[10] After a 5-min incubation period in the presence of glucagon and/or phosphodiesterase inhibitors, the tissue was removed from the incubation flasks and homogenized in 3 ml of cold 0·154 M KF (adjusted to pH 6·7 and 0·2 M Tris). The assay involved the incorporation of glucose 1-phosphate into a primer of glycogen with the release of inorganic phosphate. The reaction mixture contained 0·1 ml of the homogenate, glucose 1-phosphate (7·5 mg/ml), and glycogen (3 mg/ml) in a final volume of 1 ml. This mixture was incubated 10 min at 37°, and 1 ml of 5% trichloroacetic acid was added. This was then centrifuged, and 1 ml of the supernatant was taken for determination of Pi.[11]

Phosphodiesterase activity and inhibition constants. Phosphodiesterase was assayed by the method of Huang and Kemp[12] in the 100,000 *g* supernatant fraction of rat liver. The tissue was homogenized in 3 vol. containing 0·25 M sucrose, 1 mM phosphate buffer (pH 6·8) and 0·1 mM EDTA. After centrifugation for 1 hr at 100,000 *g* in a Beckman model L refrigerated centrifuge, an aliquot of the supernatant was incubated in a reaction mixture containing Tris Cl, pH 7·4 (50 mM), $MgSO_4$ (5 mM), cyclic AMP-^{3}H (0·03 μCi), and varying amounts of cyclic AMP. The final volume was 0·4 ml. The mixture was incubated at 37° for sufficient time to allow not more than 20 per cent of the cyclic AMP to be hydrolyzed (5–20 min). The reaction was terminated by boiling for 3 min; the mixture was cooled to 37° and 100 μg snake venum (*Crotales atrox*) was added and the incubation continued for 10 min. Adenosine-^{3}H was separated from unreacted cyclic AMP-^{3}H on DEAE Sephadex A-25

columns (0·5 × 4·5 cm) by the method of Huang and Kemp.[12] Samples were counted in a Packard Tri-Carb liquid scintillation counter (model 3375). Inhibitor constants were determined for both high-K_m and low-K_m phosphodiesterase activity by measuring velocity at two substrate concentration ranges (0·2 to 3 μM and 5 to 1000 μM) in the presence and absence of two concentrations of inhibitor. Data were plotted by the Lineweaver–Burk plot of 1/v vs 1/[s]. Slopes (K_m/V_{max}) and y intercepts ($1/V_{max}$) were obtained by use of BMD Biomedical Computer Program BMD05R.[13] K_i values were calculated according to the equation for competitive inhibition:

$$K'_m = K_m(1 + [I]/K_i)$$

where K'_m is the apparent affinity constant in the presence of inhibitor and [I] is the inhibitor concentration.

Materials. Glucagon (Lot No. 258 D30 138-4) and papaverine HCl were supplied courtesy of Dr. Walter Shaw, Eli Lilly & Co., Indianapolis, Ind. The SQ 20,009 [1-ethyl-4-(isopropylidenehydrazine)-1H-pyrazole-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester HCl] was kindly supplied by Dr. S. Hess (E. R. Squibb & Sons, New Brunswick, N.J.). Theophylline was purchased from Merck & Co. (Rahway, N.J.). Dibutyryl (DB) cyclic 3′,5′-AMP (N^6,O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate) was purchased from Boehringer Mannheim Corp. (Mannheim, Germany). Cyclic 3′,5′-AMP was purchased from Sigma Chemical Co. (St. Louis, Mo.), and ^{3}H-cyclic 3′,5′-AMP from New England Nuclear (Boston, Mass.).

RESULTS AND DISCUSSION

It is virtually impossible to demonstrate activation of phosphorylase in freshly prepared rat liver slices, since most of the enzyme is in the active form.[14] However, activity rapidly fell during a brief incubation period to a mean activity of 65 ± 3·5 μmoles Pi/g/10 min, and partial activation *in vitro* was achieved by adding glucagon (Fig. 1). Maximum activation was achieved within 5–10 min, and similar maximal responses were obtained in the presence of 10^{-6} M glucagon or 10^{-4} M DB cyclic AMP (Fig. 2). In comparison, epinephrine (10^{-6}–10^{-5} M) produced appreciably less activation of the enzyme. Previous reports have also indicated that epinephrine is much less effective than glucagon in activation of liver adenylate cyclase[15] and elevating cyclic 3′,5′-AMP levels.[16,17]

The three phosphodiesterase inhibitors tested had quite different effects on liver phosphorylase activity (Fig. 3). SQ 20,009 was without significant effect at any of the concentrations tested. At 10^{-3} M, theophylline significantly inhibited phosphorylase activity, while papaverine, at 10^{-4} and 5×10^{-4} M, produced marked elevation in phosphorylase activity.

These agents, papaverine, theophylline and SQ 20,009, were tested for their ability to inhibit phosphodiesterase activity in the 100,000 *g* supernatant fraction from rat liver. Due to the reports of a number of workers concerning the presence of high- and low-K_m phosphodiesterase activities in various tissues,[12,18,19] phosphodiesterase activity was assayed at both high and low substrate concentration ranges. Activity with a low K_m was measured at cyclic 3′,5′-AMP concentrations between 0·2 and 3 μM, and activity with a high-K_m was assayed at 5–1000 μM cyclic 3′,5′-AMP. Apparent K_m values for the two activities as well as inhibitor constants for the three phosphodiesterase inhibitors were calculated from Lineweaver–Burk plots of the

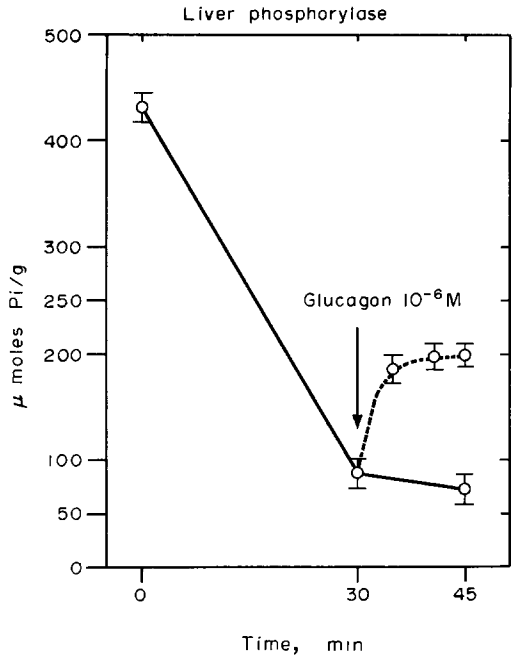

FIG. 1. Phosphorylase activity in liver slices has been plotted as a function of incubation time. Units are expressed as μmoles Pi released from glucose 1-phosphate/g wet liver/10-min assay. Control values are indicated by the solid line. Activation after addition of 10^{-6} M glucagon is indicated by the broken line. All points represent the mean ± S.E. of eight observations.

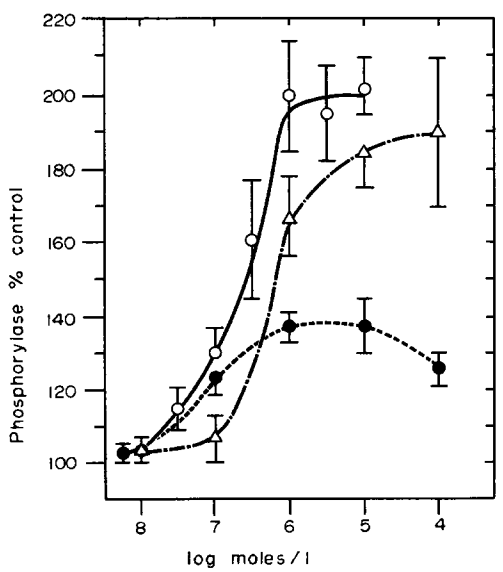

FIG. 2. Phosphorylase activity as per cent of incubated control was determined in liver slices after stimulation with glucagon (○), DB cyclic AMP (△) or epinephrine (●). Slices were incubated in the presence of the drug for 10 min prior to homogenization in 0·154 M buffered KF and assayed for phosphorylase activity. Each point represents the mean ± S.E. of six or more observations.

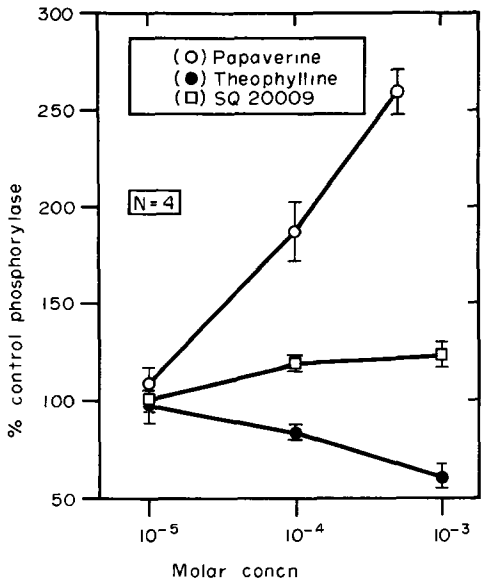

FIG. 3. Effects of three phosphodiesterase inhibitors were tested in liver slices after a 10-min incubation period. Values are plotted as per cent of control and represent mean ± S.E. for four observations.

data. These results are summarized in Table 1. In all cases, the inhibition by the phosphodiesterase inhibitors was found to be competitive.

Results of these studies, in agreement with Russell *et al.*,[20] suggest the presence of two different phosphodiesterase activities, one with an apparent K_m of 200 μM and the other with an apparent K_m of 3 μM. In both cases, theophylline was the least potent inhibitor of phosphodiesterase activity, followed by papaverine, with SQ 20,009 being the most potent inhibitor of both enzymatic activities.

Studies in which changes in cyclic AMP levels and phosphorylase activity were measured revealed that glucagon can produce a significant increase in phosphorylase activity with minimal or no detectable changes in cyclic AMP levels (Table 2). This is consistent with the results of Park *et al.*[21] who demonstrated that very small changes in cyclic AMP levels result in full activation of phosphorylase in liver. This

TABLE 1. INHIBITOR CONSTANTS FOR THEOPHYLLINE, PAPAVERINE AND SQ 20,009*

Phosphodiesterase	Inhibitor constants K_i High K_m activity† (μM)	Low K_m activity‡ (μM)
Theophylline	6000	560
Papaverine	250	10
SQ 20,009	70	4

* Inhibitor constants determined for Lineweaver–Burk plots as described in Methods. High K_m activity was determined at cyclic 3',5'-AMP concentrations between 5 and 1000 μM; low K_m activity at cyclic 3',5'-AMP concentrations between 0·2 and 3 μM.

† High K_m activity: K_m = 200 μM.

‡ Low K_m activity: K_m = 3 μM.

TABLE 2. EFFECTS OF GLUCAGON AND PHOSPHODIESTERASE INHIBITORS ON PHOSPHORYLASE ACTIVITY AND CYCLIC 3',5'-AMP LEVELS

Additions	Phosphorylase activity (% control)	N	Cyclic 3',5'-AMP levels (pmoles/mg wet wt)	N
Control	100 ± 9*	8	0·45 ± 0·04*	17
Glucagon (10^{-6} M)	172 ± 18†	8	0·60 ± 0·06†	16
Papaverine (5×10^{-4} M)	178 ± 15†	8	0·53 ± 0·10	6
Theophylline (10^{-3}M)	73 ± 9†,‡	8	0·51 ± 0·14	5
SQ 20,009 (10^{-3} M)	105 ± 5‡	8	0·44 ± 0·02	6
Glucagon + papaverine	165 ± 12†	8	3·16 ± 0·71†,‡	6
Glucagon + theophylline	130 ± 14†,‡	8	1·15 ± 0·16†,‡	5
Glucagon + SQ 20,009	165 ± 20†	8	1·93 ± 0·58†,‡	6

* Mean ± S.E.M.
† Value is significantly different from control ($P < 0·05$).
‡ Value is significantly different from value in presence of glucagon ($P < 0·05$).

would also explain why the phosphodiesterase inhibitors significantly augmented the ability of glucagon to elevate cyclic AMP levels but failed to increase the effect of glucagon on phosphorylase activity (Table 2).

On first inspection, it seems possible to explain the ability of papaverine to activate glucogen phosphorylase on the basis of phosphodiesterase inhibition. This, however, is an unlikely explanation, as the more potent phosphodiesterase inhibitor, SQ 20,009, failed to increase phosphorylase activity and the classical phosphodiesterase inhibitor, theophylline, inhibited phosphorylase. It cannot be assumed that an agent which inhibits phosphodiesterase in a supernatant preparation also inhibits the enzyme in the intact cell. It would appear, however, that all three agents tested were capable of producing inhibition of phosphodiesterase in the intact cell, as they all augmented the ability of glucagon to stimulate cyclic AMP accumulation in the liver slice preparation (Table 2). Based on the observations that papaverine stimulated phosphorylase in liver slices while SQ 20,009 was without effect, it seems unlikely that papaverine produced this response secondary to its ability to inhibit phosphodiesterase. It could be argued that SQ 20,009 did stimulate phosphorylase activity in liver slices, but this effect was masked by a more direct, nearly equal, inhibition of the phosphorylase reaction. This does not seem to be the case, as SQ 20,009 had no effect on phosphorylase activity in liver homogenates (see below).

The exact mechanism by which papaverine stimulated phosphorylase is unknown. It is known, however, that papaverine inhibits liver mitochondrial electron transfer (R. A. Harris, personal communication). The resulting accumulation of 5'-AMP could possibly lead to an activation of phosphorylase.

In these studies, theophylline produced a significant decrease in phosphorylase activity in liver slices. It has been demonstrated that theophylline increases phosphorylase phosphatase activity,[22] thus causing a more rapid inactivation of phosphorylase. In our own experience, we have found that theophylline inhibited purified liver phosphorylase (which was obtained courtesy of Dr. Joel Hardman, Vanderbilt University) and reduced phosphorylase activity, in liver homogenates even in the presence of isotonic fluoride, an inhibitor of phosphorylase phosphatase (Fig. 4). This action of theophylline would apparently not involve phosphorylase phosphatase,

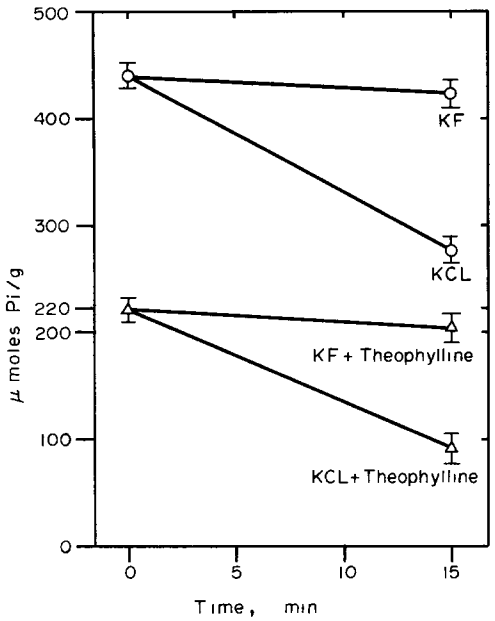

FIG. 4. Rat liver was homogenized in 0·154 M buffered KF or KCl in the presence or absence of 10^{-3} M theophylline. Phosphorylase activity was assayed immediately, or after a 15-min incubation. Phosphorylase values are expressed as μmoles Pi released from glucose 1-phosphate/g wet liver/10-min incubation. Each value represents the mean ± S.E. for six observations.

because the inhibition was immediate and phosphorylase activity was stable in isotonic fluoride either with or without theophylline. In the absence of fluoride, phosphorylase activity decays rapidly, presumably the results of the phosphorylase phosphatase reaction. In similar experiments, neither papaverine nor SQ 20,009 had any effect on phosphorylase activity in liver homogenates prepared either in the presence or absence of fluoride.

It is naive to think that a pharmacological agent has only one action. Theophylline, SQ 20,009 and papaverine undoubtedly have many actions in addition to inhibition of phosphodiesterase. The results of this study and other similar studies[5,23,24] suggest that great care should be taken in assuming any cause and effect relationships between a drug's ability to inhibit phosphodiesterase and to produce a metabolic response.

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