5-Membered Heterocyclic Compounds: A New Class of Hypocalcemic Agents[1] (35075)

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2-Thiophenecarboxylic acid (2-TCA) is a hypoglycemic and antilipolytic agent (1–3). In the course of studies of a variety of agents which affect the concentration of calcium in serum, we discovered that 2-TCA produced marked hypocalcemia and hypophosphatemia in the rat (4). After a single sc injection (2 mmoles/kg body wt, equivalent to about 30 mg/rat), the hypocalcemic effect is rapid in onset (it occurs in less than 30 min), large in magnitude (a fall of total serum calcium of 25%), and moderately short in duration (4–6 hr). The hypocalcemic action of 2-TCA is very similar to that observed after treatment of rats with calcitonin, a natural polypeptide hormone (5, 6); however 2-TCA is effective by oral administration. *In vivo* studies previously reported (4) have eliminated thyroid and parathyroid glands, kidneys, and pancreas as essential mediators of the hypocalcemic effect of 2-TCA.

Because 2-TCA is a simple compound and gave no evidence of toxicity in the rats even at six times the maximally effective hypocalcemic dose, we concluded that further studies of the relationships between chemical structure and biological activity and of its mode and site of action might lead to a new group of pharmacologically useful agents. In this report, we present evidence that several thiophene derivatives possessing a carboxylic acid or carboxaldehyde group and 2-pyrrolecarboxylic acid, an analog of 2-TCA, have hypocalcemic activity in the rat and that the prototype agent, 2-TCA, acts directly on the skeleton to inhibit mineral transfer from bone to the surrounding medium.

Materials and Methods. The compounds (listed in Table I) were obtained from Eastman Organic Chemicals, Rochester, N.Y. (thiophene, 2-thiopheneethanol and 2-thiophenecarboxaldehyde); K & K Laboratories, Inc., Plainview, N.Y. (2-thiophenecarbonyl chloride and 3-thiophenecarboxylic acid); and Aldrich Chemical Co., Inc., Milwaukee, Wis. (the remaining 5 compounds). The compounds were used without further purification, except 2-TCA which was recrystallized twice from ethanol solution.

Male Holtzman rats at 6 weeks of age (120–150 g) were used in these experiments. They were fed a low calcium diet (5) for 2 or 3 days before each experiment. Blood samples of 4 ml or more from each rat were taken by cardiac puncture, and the concentration of calcium in serum was determined by the method of Copp (7). The data in each experiment were subjected to an analysis of variance. The standard errors were calculated from the residual error term of that analysis.

In one experiment, bilateral nephrectomy was performed in two groups of 24 rats. One group of animals received a sc injection of 2-TCA (2 mmoles/kg) before, at the time of, and at various times after operation, while the control group received saline injections. Blood samples were taken by cardiac puncture 2 hr after the injection in groups of four rats each and serum phosphorus (8) as well as serum calcium was determined.

Bone culture. Calvaria from 5-day-old

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TABLE I. Hypocalcemic Effects of Thiophene and Related Compounds.[a]

Compound	Dose (mg/rat)	Serum calcium (mg/100 ml)[b] Exp. 1	Exp. 2
Saline (control)		10.3	10.1
Thiophene	100	10.2	—
2-Methylthiophene	200	—	10.2
2-Thiophenecarbonyl chloride	100	10.1	—
2-Thiopheneethanol	100	10.3	—
2-Thiophenecarboxaldehyde	100	8.2	—
5-Methyl-2-thiophenecarboxaldehyde	100	—	8.6
2-Thiophenecarboxylic acid (2-TCA)	30	8.4	—
	40	—	7.5
5-Methyl-2-thiophenecarboxylic acid	40	—	8.2
3-Thiophenecarboxylic acid	30	8.5	—
	40	—	7.5
2-Pyrrolecarboxylic acid	40	—	8.2

[a] Solid compounds were injected in neutral aqueous solutions and liquid compounds were injected directly in saline suspension. Rats were bled 1½ hr after sc injection (1 ml/rat).

[b] Mean values, four rats/group. The SE in Exps. 1 and 2 were ± 0.14 and 0.21, respectively.

mice (Charles River CD-1) were removed aseptically and the frontal and parietal portions were fixed to a coverslip by a plasma clot (9). Each coverslip with a calvarium was placed in a Leighton tube containing 2 ml of medium composed of 80% horse serum and 20% Gey's balanced salt solution as well as 10,000 U of penicillin, 10 mg of streptomycin, and 0.25 mg of phenol red per 100 ml (9). To stimulate bone resorption, we added 0.5 USP U of bovine parathyroid hormone (PTH)[4] to each ml of medium. The sodium salt of 2-TCA was dissolved in Gey's solution before incorporation into culture medium. Tubes were gassed with a mixture of 50% oxygen and 50% nitrogen and incubated in a roller-drum apparatus at 37°. Medium was changed every 48 hr. The calcium concentration in the medium before and at the end of each culture period was measured (7). At the end of 6 days, the bones were fixed in 10% neutral formalin and stained with hematoxylin and eosin or by von Kossa's reaction for histological examination (9).

In one experiment, one group of control bones (four bones/group) and four groups of bone treated with PTH were preincubated for 48 hr. Three groups of the hormone-treated bones were then incubated in medium containing 2-TCA (1 mg/ml) for limited periods (2, 4, and 6 hr, respectively). All bones were then washed thoroughly with fresh medium without 2-TCA. Experimental cultures were incubated again in 2-TCA-free medium containing PTH for the remainder of the experiment; two control cultures were incubated in medium either with or without hormone. Calcium and phosphorus concentrations of all media were measured.

Results and Discussion. Structure-activity relationships. We have tested nine thiophene compounds and one pyrrole analog. The results in Table I show that 5 thiophene derivatives and 2-pyrrolecarboxylic acid have hypocalcemic activity. None of the compounds which were hypocalcemic produced any overt evidence of acute toxicity in the rats at the dose levels tested. All the active compounds contain either a carboxylic acid or carboxaldehyde group; however, its position is not limited to carbon 2 of the thiophene ring, since 3-thiophenecarboxylic acid is as active as 2-TCA. If the pyrrole ring is substituted for the thiophene ring, as in 2-pyrrolecarboxylic acid, hypocalcemic activity is retained.

Action of 2-TCA in nephrectomized rats.

[4] Supplied by Eli Lilly & Co., Indianapolis, Ind.

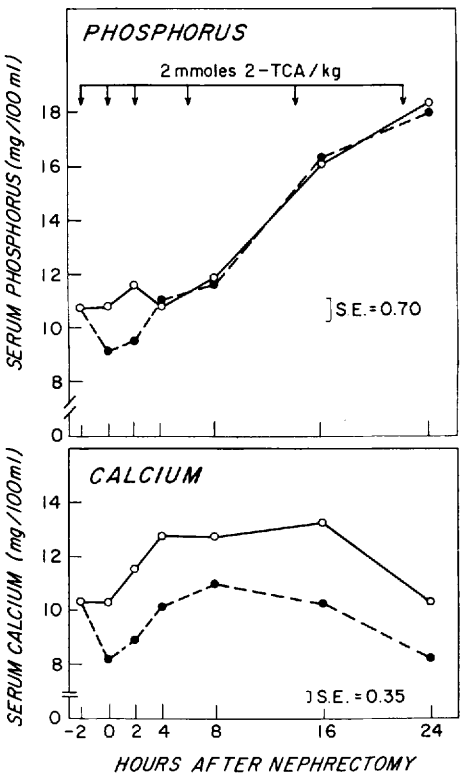

FIG. 1. Hypocalcemic and hypophosphatemic effects of 2-TCA in nephrectomized rats. Each point gives the mean value of four rats. Rats were sacrificed 2 hr after injection. O—O = saline control, ●—● = 2-TCA.

2-TCA prevented the acute hypercalcemia that follows complete nephrectomy in the rat (Fig. 1). The hypophosphatemic effect of 2-TCA, however, was only observed during the first 2 hr after nephrectomy; thereafter, saline-injected and 2-TCA-treated nephrectomized rats had the same serum phosphorus levels (Fig. 1).

Direct effect of 2-TCA on bone in culture. Figure 2 shows net movement of calcium out of medium into bone in control cultures and into medium from PTH-treated bones. The results also show that 2-TCA inhibited the PTH-stimulated calcium transfer from bone to medium. A clear relationship between the dose of 2-TCA used and the extent of inhibition was observed. Examination of histological sections of bone samples taken after 3 days of treatment with 2-TCA revealed no morphological evidence of toxicity when the concentration of 2-TCA was 0.5 mg or less in 2 ml culture medium. Biochemical studies of the effect of 2-TCA on bone formation using ^{3}H-proline incorporation into bone collagen are in progress.

2-Pyrrolecarboxylic acid has been found to have the same direct effect as 2-TCA on bone cultures, suggesting that this entire group of agents has direct effects on bone.

Measurements of concomitant changes

TABLE II. Effects of 2-TCA on Calcium and Phosphorus in Medium of Mouse Calvaria in Tissue Culture.

Group	Days of incubation[a] 0–2 ΔCa (μg/calvarium)	0–2 ΔP (μg/calvarium)	2–4 ΔCa (μg/calvarium)	2–4 ΔP (μg/calvarium)	Net ΔCa/ΔP[c]
Control	−5	+11	−30	−13	2.3
PTH alone (0.5 U/ml)	+23	+24	+42	+20	2.1
PTH + 2-TCA, 2 hr[b]	—	—	+12	+10	3.0
PTH + 2-TCA, 4 hr[b]	—	—	+10	+10	3.2
PTH + 2-TCA, 6 hr[b]	—	—	+6	+11	4.0

[a] Changes (Δ) of Ca and P are expressed as mean values of four calvaria in each group (SE = ±4 μg for both Ca and P). Negative values represent minerals taken up by bone from medium and positive values represent minerals released from bone to medium.

[b] Medium containing 2-TCA (1 mg/ml) was added to culture at the beginning of incubation day 3 for 2, 4, or 6 hr, respectively, and then was replaced with medium containing PTH alone for the remainder of the incubation period.

[c] Net changes of Ca and P for 2-TCA-treated groups were calculated as differences from the PTH-alone group.

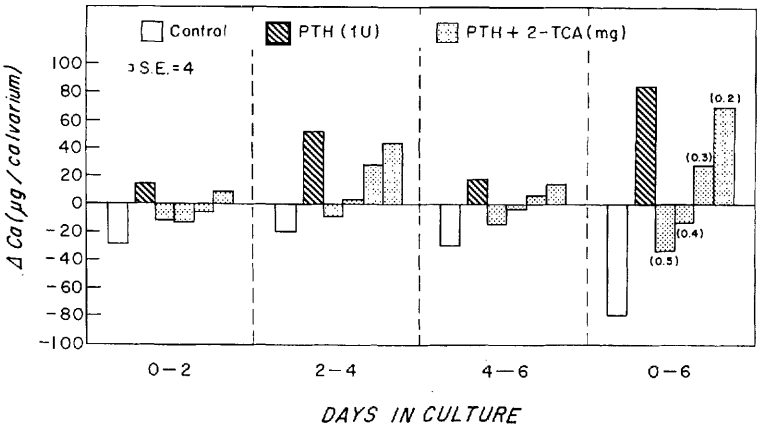

FIG. 2. Effects of PTH and PTH + 2-TCA on the concentration of calcium in medium of bone cultures. The initial level of calcium in medium was about 10 mg/100 ml. The bars give mean changes (Δ) of calcium in medium per bone in a group of four bones. Negative values indicate calcium taken up by bone from medium, and positive values indicate calcium transferred from bone to medium. Media were changed every 48 hr for a total of 6 days. The first three panels give the results in consecutive 2-day periods. The last panel (0–6 days) gives the cumulative mean changes. The doses of 2-TCA were 0.2, 0.3, 0.4, and 0.5 mg/2 ml culture medium.

of calcium and phosphorus in culture medium (Table II) are consistent with the previous finding (Fig. 1) that 2-TCA has a greater effect on serum calcium than it has on serum phosphorus. In our culture system, bones do not respond maximally to PTH until days 3 and 4 of incubation (Fig. 2). During the first 2 days of incubation, control bones take up calcium from medium (Fig. 2, Table II) and release phosphorus to medium (Table II), and PTH-treated bones release both calcium and phosphorus into medium (Fig. 2 and Table II). During the second 2 days of incubation, control bones take up both calcium and phosphorus from medium and PTH-treated bones release both calcium and phosphorus to medium (Table II). It is noteworthy that the ratio of Δ calcium to Δ phosphorus in medium in both control and PTH-treated bones was 2.1 to 2.3 which approximates the ratio of calcium to phosphorus in hydroxyapatite ($3Ca_3(PO_4)_2 \cdot Ca(OH)_2$). However, when compared to the PTH-treated group, bones treated with 2-TCA released relatively less calcium than phosphorus into the culture medium (Table II).

The inhibitory effects of 2-TCA on calcium and phosphorus transfer on bones in culture can be demonstrated after periods of exposure as brief as 20 min (unpublished data). After the removal of 2-TCA from culture medium, bones recover quickly their responsiveness to PTH. These findings show that the action of 2-TCA on bone is not irreversible.

Summary. Five thiophene derivatives and 2-pyrrolecarboxylic acid have marked hypocalcemic activity in rats. In nephrectomized rats, 2-TCA has a greater effect on serum calcium than it has on serum phosphorus. In a tissue culture system, 2-TCA inhibits directly PTH-stimulated transfer of calcium and phosphorus from bone. These effects of 2-TCA are rapid, reversible, and dose-related.

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1. Fang, V. S., Arch. Int. Pharmacodyn. Ther. **176**, 193 (1968).

2. Fang, V. S., Foye, W. O., Robinson, S. M., and Jenkins, H. J., J. Pharm. Sci. **57**, 2111 (1968).

3. Fang, V. S., Arch. Int. Pharmacodyn. Ther. **178**, 315 (1969).

4. Lloyd, W., Fang, V. S., Wells, H., and Tashjian, A. H., Jr., Endocrinology **85**, 763 (1969).

5. Hirsch, P. F., Gauthier, G. F., and Munson, P. L., Endocrinology **73**, 244 (1964).

6. Hirsch, P. F., and Munson, P. L., Physiol. Rev. **49**, 548 (1969).

7. Copp, D. H., J. Lab. Clin. Med. **61**, 1029 (1963).

8. Greenberg, B. G., Winters, R. W., and Graham, J. B., J. Clin. Endocrinol. Metab. **20**, 364 (1960).

9. Goldhaber, P., Dent. Res. **45**, 490 (1966).

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