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EFFECTS OF FORSKOLIN ON BONE

STIMULATION OF CYCLIC AMP ACCUMULATION AND CALCIUM EFFLUX FROM CHICK EMBRYO TIBIAE IN ORGAN CULTURE

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Abstract—Forskolin (FSK), an activator of adenylate cyclase in many tissues, was investigated to determine its potential for studying cyclic AMP-mediated, physiological events in bone. Tibiae from 14-day chick embryos were cultured in modified Eagle's medium for up to 8 hr, and the cyclic AMP and Ca^{2+} efflux responses to parathyroid hormone (PTH) and FSK were observed. FSK caused a concentration-related increase in tissue cyclic AMP, with 10^{-6} M FSK producing a cyclic AMP response of similar magnitude to that elicited by 10^{-6} M PTH (1–34). However, while 10^{-6} M PTH was the maximally-activating PTH concentration, a further elevation was produced by 10^{-5} M FSK. Chick embryo tibiae cultured for 8 hr in the presence of PTH exhibited a concentration-related stimulation of Ca^{2+} efflux up to 10^{-6} M PTH. When this physiological effect was examined in bones cultured in the presence of FSK, a biphasic response to increasing FSK concentrations was found; Ca^{2+} efflux was stimulated by 10^{-6} M and 3×10^{-6} M FSK, but not by 10^{-7} M or 10^{-5} M FSK. In summary, concentrations of FSK, which produced tissue cyclic AMP accumulation similar to that produced by physiologically effective PTH concentrations, also resulted in net Ca^{2+} efflux from bone. These results indicate that FSK may be a useful agent for studying the role of cyclic AMP in the response of bone to hormones.

Forskolin (FSK), a diterpene compound extracted from the plant roots of *Coleus forskohlii*, is of current interest as a pharmacological tool for the investigation of the intermediary role of cyclic AMP in hormone-stimulated events. FSK has been shown to activate the adenylate cyclase enzyme complex of cell membrane preparations and to elevate cyclic AMP levels in whole cells from a variety of tissues [1–5]. The FSK activation of adenylate cyclase from different sources occurs rapidly, reversibly, and in a dose-dependent fashion [1]. Whereas stimulation of adenylate cyclase by sodium fluoride, guanyl-5′-yl imidodiphosphate, cholera toxin, or hormone agonists requires the guanine nucleotide binding (G) subunit, stimulation by FSK does not [1, 5]. However, a recent report indicates that FSK stimulation of adenylate cyclase is enhanced in the presence of the G subunit [5]. Additionally, FSK can potentiate hormonally-induced elevations of cyclic AMP, either by increasing the magnitude of response or by increasing the apparent potency of the agonist [1–5].

The present work was undertaken to investigate the potential usefulness of FSK for studying cyclic AMP-mediated events in bone. Although parathyroid hormone (PTH) is known to stimulate bone adenylate cyclase activity [6] and to increase bone cell cyclic AMP content [7], little is known about cyclic AMP regulation of the physiological effects of PTH in bone.

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Tibiae from embryonic chicks have been shown to remain viable and grow in organ culture for up to 3 days [8]; in this system, PTH has been shown to stimulate bone Ca^{2+} efflux [9], decrease alkaline phosphatase activity [8, 10], and increase both ATPase activity [8] and lactate release [10]. In the present study, tibiae from 14-day chick embryos were used to compare the effects of PTH and FSK on cyclic AMP accumulation and Ca^{2+} efflux.

METHODS AND MATERIALS

Modified Eagle's minimum essential medium [9], buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and containing 0.1% bovine serum albumin (BSA), 1 mM phosphorus, and 0.5 to 2.2 mM Ca^{2+}, as indicated, was freshly prepared for all experiments.

Cyclic AMP accumulation experiments. Tibiae from 14-day chick embryos were harvested with the periostea intact and the epiphyseal cartilage removed. All bones were pooled and maintained in ice-cold medium, pH 7.4, containing 0.1% BSA, 1 mM phosphorus and 1.8 mM Ca^{2+}. The bones were then incubated in medium adjusted to 0.5, 1.8 or 2.2 mM Ca^{2+}, as indicated, in a metabolic shaker at 37° for a 30-min equilibration period. Next, tibiae were randomly transferred to the appropriate Ca^{2+} medium to which PTH, FSK, or control vehicle (3×10^{-6} N acetic acid or 0.1% ethanol respectively) had been added and further incubated for 2.5 min to 8 hr. At the end of this test incubation period, each bone was immediately placed in 0.05 M sodium acetate buffer, pH 4.0, maintained at 100° for 5 min.

The tissue was homogenized with a Tissumizer (Tekmar Co., Cincinnati, OH) and centrifuged. The supernatant fraction was collected and stored at −20° until assay for cyclic AMP by radioimmunoassay. No phosphodiesterase inhibitors were added to the media in order to measure bone cyclic AMP accumulation under the same conditions used to evaluate Ca^{2+} efflux.

Ca^{2+}efflux experiments. Tibiae from 14-day chick embryos were dissected as described above. For these experiments, however, the tibiae from each embryo were paired between control and experimental conditions. Two or three tibiae were placed in a capped glass vial containing 0.25 ml/bone of control medium, pH 7.4, at the indicated Ca^{2+} concentration; the corresponding two or three bones were placed in medium of the appropriate Ca^{2+} concentration to which either PTH or FSK had been added. Five or six vials per condition were incubated for a total of 8 hr at 37° in a metabolic shaker. Medium from each vial was collected at 2, 4, 6, and 8 hr and replaced with fresh medium. Medium samples were assayed for Ca^{2+} and phosphorus. Net ion fluxes were calculated as the differences between total medium ion concentrations in the presence of and in the absence of bones. The differences were divided by the number of bones per vial to yield net ion efflux expressed as μg/bone. A medium concentration of 1.8 mM Ca^{2+} corresponds to 18 μg Ca^{2+}/bone; each 1 μg/bone of Ca^{2+} efflux increased the medium Ca^{2+} concentration by 6%. In some experiments, paired control and treated tibiae were removed at 2-hr intervals for the determination of cyclic AMP levels in the tissue.

Analytical methods. Cyclic AMP was measured in bone extracts by radioimmunoassay, using the method of Steiner *et al.* [11] as modified by Harper and Brooker [12]. The concentration of total Ca^{2+} in the media was measured by atomic absorption spectrophotometry (Perkin Elmer, Norwalk, CT). Medium was diluted with $LaCl_3$ to eliminate interference by phosphate. The concentration of phosphorus was estimated from inorganic PO_4 assayed colorimetrically by the method of Chen *et al.* [13].

Statistical methods. For cyclic AMP accumulation experiments, the level of significance of the difference between means of experimental and control groups was determined using a one-tailed Student's *t*-test. For Ca^{2+} efflux experiments, a one-tailed *t*-test for paired data was employed. Because this test of significance is based on the differences between pairs of bones, no inferences as to the level of significance can be made from the S.E.M. Values of S.E.M. are included only as an indication of experimental variation. A value of $P < 0.025$ was considered significant.

Materials. All reagents used were of analytical quality and were purchased from standard suppliers. Parathyroid hormone (bovine synthetic 1–34) was purchased from Peninsula Laboratories Inc. (San Carlos, CA); the hormone was reconstituted in 0.1 N glacial acetic acid and stored at −20°. Forskolin was purchased from Calbiochem-Behring (La Jolla, CA); the drug was reconstituted in 95% ethanol and stored at −20°.

Fertilized White Leghorn chicken eggs were obtained from the Department of Poultry Husbandry, University of Missouri (Columbia, MO); they were incubated for 14 days in Marsh-Rollex incubators.

RESULTS

Bone cyclic AMP response to PTH and FSK. Tibiae were incubated for up to 8 hr in either control medium or in medium containing 10^{-6} M PTH (a maximally-activating concentration) or 10^{-6} M FSK. This FSK concentration most closely duplicated the maximal cyclic AMP response to PTH in this system (Fig. 1). Table I shows that 10^{-7} M FSK caused only a 1.5- to 2-fold increase in tibia cyclic AMP content while 10^{-5} M FSK caused a very large and rapid elevation. The data in Fig. 1 indicate that PTH stimulated a rapid accumulation of cyclic AMP in tibiae with a sharp, peak response occurring at 15 min and gradually returning toward control levels during the subsequent 2 hr. The maximal response to 10^{-6} M FSK was more prolonged, but by 4 hr PTH- and FSK-treated bones both contained approximately twice the control concentration of cyclic AMP.

Dose-related Ca^{2+} efflux response to PTH and FSK. The data in Fig. 2 show that 10^{-9} M PTH did not stimulate net Ca^{2+} efflux from 14-day embryo tibiae above basal levels during the 8-hr incubation. However, from 10^{-8} to 10^{-6} M PTH, a concentration-related increase in net bone Ca^{2+} efflux occurred.

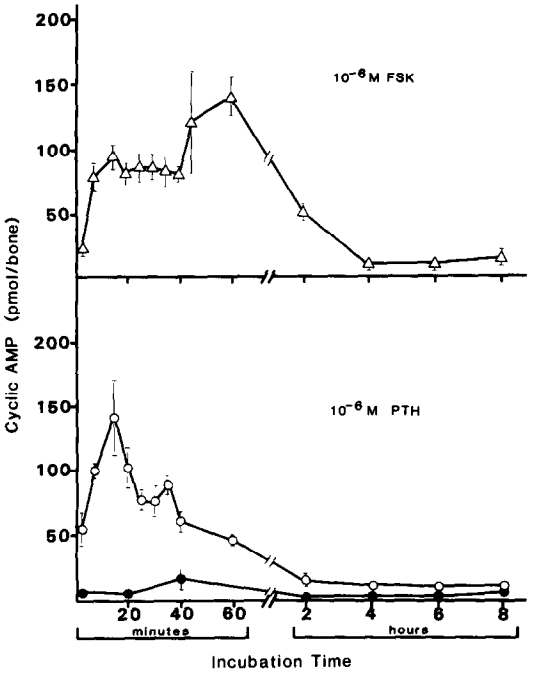

Fig. 1. Cyclic AMP accumulation by tibiae from 14-day chick embryos during an 8-hr incubation with 10^{-6} M FSK (upper panel) or 10^{-6} M PTH (1–34) (lower panel). Medium Ca^{2+} and phosphorus concentrations were 1.8 and 1 mM respectively. PTH- and FSK-stimulated bones contained significantly ($P < 0.01$) more cyclic AMP than bones in control medium (●——●) at all time points except 8 hr.

Table 1. Tibia cyclic AMP accumulation in response to FSK*

	Cyclic AMP accumulation			
	Incubation time (min)			
	2.5	20	30	40
Control	5.9 ± 1.2	4.4 ± 0.5		5.2 ± 0.7
10^{-7} M FSK	8.1 ± 0.9	8.8 ± 1.3†	9.1 ± 0.8‡	8.1 ± 0.1†
10^{-5} M FSK	39 ± 2‡	696 ± 100‡	453 ± 14‡	472 ± 19‡

* Each determination represents the mean ± S.E. of three to four bones.
† $P < 0.025$ relative to control.
‡ $P < 0.01$ relative to control.

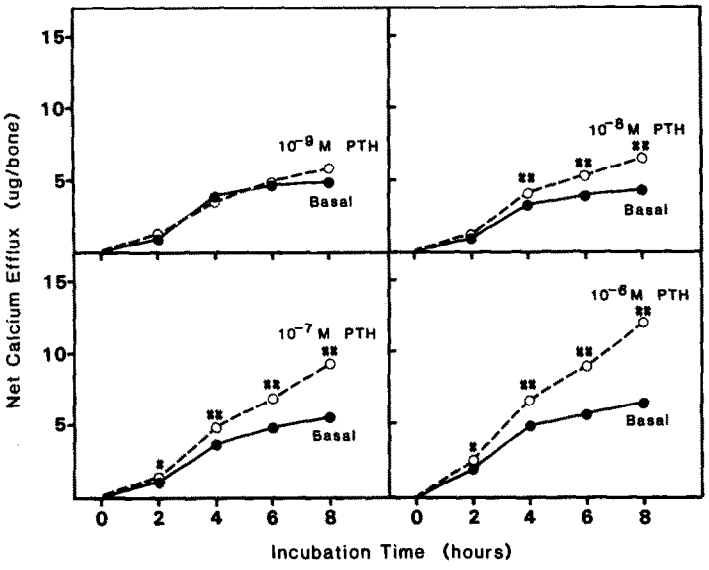

Fig. 2. PTH-stimulated net Ca^{2+} efflux from bones during an 8-hr incubation in medium containing 1.8 mM Ca^{2+}, 1 mM phosphorus, and the indicated concentration of PTH or appropriate volume of vehicle (basal). There was no effect of PTH on net phosphorus efflux by any concentration of PTH. Key: (*) $P < 0.025$ and (**) $P < 0.01$ relative to paired bones under basal conditions; N = 5–6 pairs per group.

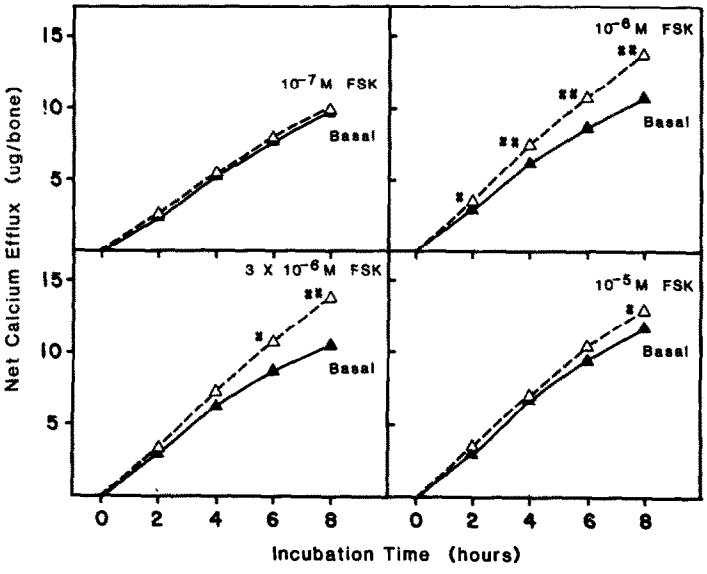

Fig. 3. FSK-stimulated Ca^{2+} efflux from 14-day chick embryo tibiae. See legend to Fig. 2 for details. Key: (*) $P < 0.025$ and (**) $P < 0.01$ relative to paired bones under basal conditions; N = 5–6 pairs per group.

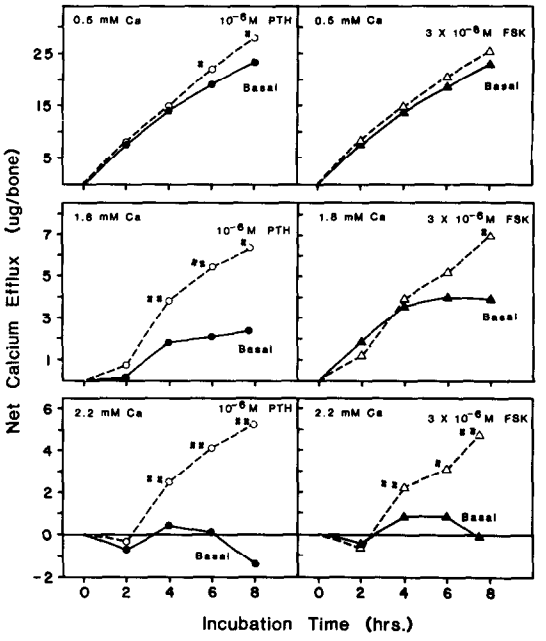

Fig. 4. Medium Ca^{2+} concentration and net Ca^{2+} efflux of tibiae cultured for 8 hr in medium containing 1 mM phosphorus and 10^{-6} M PTH or 3×10^{-6} M FSK. Key: (*) $P < 0.025$ and (**) $P < 0.01$ relative to paired bones cultured in control medium; N = 4–5 pairs per group.

No PTH stimulation of net phosphorus efflux was observed in this or subsequent experiments (data not shown). These data corroborated earlier findings of Ramp and McNeil using PTH [9] and provided a basis for comparison of the effects of FSK in this system.

Figure 3 shows that 10^{-6} and 3×10^{-6} M FSK also stimulated net Ca^{2+} efflux from bones, while 10^{-7} and 10^{-5} M FSK were not effective in increasing net Ca^{2+} efflux. The lack of Ca^{2+} efflux response to 10^{-7} M FSK corresponds to the very small bone cyclic AMP response to that FSK concentration (Table 1). However, exposure to 10^{-5} M FSK resulted in very large increases in bone cyclic AMP content, but only a small increase in net Ca^{2+} efflux after 8 hr. Additional experiments (data not shown) demonstrated that the control vehicle (0.1% ethanol) had no effect on basal Ca^{2+} efflux, the FSK dose–response relationship, or PTH-stimulated Ca^{2+} efflux.

Effects of medium Ca^{2+} concentration on PTH- and FSK-stimulated cyclic AMP accumulation and selective Ca^{2+} efflux. Evidence exists for the involvement of Ca^{2+}, as well as cyclic AMP, in the physiological response of bone cells to PTH. Recent studies using isolated fetal rat bone cells [14] and isolated perfused dog bone [15] have suggested that extracellular Ca^{2+} concentration might affect the magnitude of the cyclic AMP response of bone to PTH. Therefore, the effects of this cation on PTH- and FSK-stimulated cyclic AMP accumulation and Ca^{2+} efflux were evaluated in the present system. Both agents produced dose-related increases in the levels of cyclic AMP in chick embryo tibiae, but varying the medium Ca^{2+}concentration from 0.5 to 2.2 mM did not alter the cyclic AMP response to either PTH or FSK (data not shown).

Although increasing extracellular Ca^{2+} from 0.5 to 2.2 mM did not potentiate agonist-stimulated adenylate cyclase activity, the stimulation of selective Ca^{2+} efflux from bone occurred more rapidly at the high (2.2 mM) than at the lower (0.5 and 1.8 mM) Ca^{2+} concentrations for both PTH and FSK (Fig. 4). The magnitude of basal efflux from bone was less in 2.2 mM Ca^{2+} than in 0.5 mM Ca^{2+}, but the amount of stimulated Ca^{2+} efflux in response to PTH or FSK was similar in all medium Ca^{2+} concentrations (4–5 μg Ca^{2+} per bone per 8 hr). As in the previous experiments, net phosphorus efflux was not altered by the presece of 10^{-6} M PTH or 3×10^{-6} M FSK at any Ca^{2+} concentration. However, as medium Ca^{2+} was increased, the net movement of phosphorus changed from efflux to influx (data not shown). The means ± S.E.M. of the differences, shown in Fig. 4, between basal and PTH- or FSK-stimulated Ca^{2+} efflux (μg/bone) are listed in Table 2 as an indication of the experimental variation. These values are also representative of data presented in Figs. 2 and 3.

DISCUSSION

The results of this study show that, as in many other tissues [1], FSK is capable of stimulating cyclic AMP formation by chick embryo tibiae in a concentration-dependent manner. A physiological hormone response of these bones, PTH-stimulated Ca^{2+} efflux, was also activated by FSK. A significant pharmacological finding was that, unlike PTH, the physiological response to FSK was biphasic; while Ca^{2+} efflux was stimulated by 10^{-6} M FSK, neither 10^{-7} M nor 10^{-5} M FSK increased Ca^{2+} release.

It has been demonstrated previously [16] that continuous incubation of rat calvariae with PTH results

Table 2. Effect of Ca^{2+} concentration on net Ca^{2+} efflux from tibiae cultured in PTH and FSK*

	2 hr	4 hr	6 hr	8 hr
0.5 mM Ca^{2+}, 10^{-6} M PTH	0.11 ± 0.18	0.78 ± 0.20	2.91 ± 1.65	4.42 ± 1.16
3×10^{-6} M FSK	0.13 ± 0.49	0.91 ± 1.01	2.22 ± 1.24	2.20 ± 1.76
1.8 mM Ca^{2+}, 10^{-6} M PTH	0.60 ± 0.65	2.00 ± 0.38	3.34 ± 0.79	3.95 ± 1.11
3×10^{-6} M FSK	0.68 ± 0.61	0.31 ± 0.54	1.13 ± 0.75	3.02 ± 1.13
2.2 mM Ca^{2+}, 10^{-6} M PTH	0.47 ± 0.04	2.06 ± 0.33	4.06 ± 0.57	6.63 ± 0.77
3×10^{-6} M FSK	0.22 ± 0.30	1.39 ± 0.22	2.20 ± 0.53	4.80 ± 0.52

* These are the data illustrated in Fig. 4.

in desensitization of the cyclic AMP response. Consequently, upon initial exposure to PTH, bone cell cyclic AMP content rises rapidly to a peak level and then returns towards basal levels. In the present study using chick embryo tibiae, PTH also produced a rapid peak cyclic AMP response of short duration. Additionally, 10^{-6} M FSK elicited increased tissue cyclic AMP accumulation that was rapid and short-lived, although somewhat more prolonged than the response to PTH. A recent report has shown that S49 lymphoma cells became refractory to FSK stimulation of cyclic AMP production during a 4-hr exposure period [5]. This finding corresponds to the time–course of the cyclic AMP response of chick embryo tibiae to 10^{-6} M FSK in the present study.

The cyclic AMP response to 10^{-6}M PTH was the maximal response to the hormone observed in embryonic chick bone. The magnitude of the cyclic AMP response to 10^{-6} M FSK was similar to that of maximal PTH, while higher concentrations of FSK were capable of stimulating much greater cyclic AMP accumulation than PTH. At a concentration of 10^{-5} M, FSK produced a prolonged increase in tissue content of cyclic AMP which was of greater magnitude than that elicited by a maximally-stimulating concentration of PTH, yet 10^{-5} M FSK was not effective in stimulating Ca^{2+} efflux. Low concentrations of FSK (10^{-7} M) which did not increase tissue cyclic AMP content were also ineffective in increasing net Ca^{2+} efflux. This biphasic response of the physiological process to increasing levels of FSK is significant in terms of the potential of FSK as a pharmacological tool for studying the role of cyclic AMP in hormone mediated events. Extremely high tissue content of cyclic AMP may have an inhibitory effect on metabolic processes that are stimulated by lower, more "physiological" levels. Moriwaki *et al.* [17] observed stimulation of steroidogenesis by low concentrations of FSK and inhibition of steroidogenesis by high concentrations of FSK in isolated rat adrenal cells. They suggested that depletion of cellular ATP could account for the inhibition. It is possible that a similar explanation accounts for the results reported here.

The results of this study suggest that both the magnitude and duration of elevated tissue cyclic AMP levels are important in eliciting physiological responses. The ability of FSK to produce a rapid, hormone-like tissue cyclic AMP response in embryonic chick tibiae indicates that FSK may be a more useful agent than cholera toxin, cyclic AMP analogs, or phosphodiesterase inhibitors for studying the mediation of hormonal effects by cyclic AMP in bone.

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