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Forskolin Enhances Calcium-Evoked Prolactin Release from 7315c Tumor Cells without Increasing the Cytosolic Calcium Concentration

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SUMMARY

The 7315c prolactin-secreting tumor cell was used as a model of a normal pituitary cell in order to study the enhancement by adenosine 3′,5′-cyclic monophosphate (cAMP) of calcium-evoked hormone release. Forskolin and, by implication, cAMP had little effect on basal hormone release during a 10-min incubation period. Ionomycin and a high potassium concentration, treatments which enhanced the cytosolic calcium concentration, increased hormone release. When cells were exposed to forskolin prior to and during a challenge with either ionomycin or high potassium, a synergistic effect on prolactin release was observed. 8-Bromoadenosine 3′,5′-cyclic monophosphate mimicked forskolin in enhancing ionomycin-evoked prolactin release while having little effect of its own on hormone release. Forskolin did not alter the increase in cytosolic calcium concentration elicited by either ionomycin or high potassium, nor did it increase the potency of ionomycin in enhancing prolactin release. The calcium channel antagonist, D-600, did not alter ionomycin-induced release or its enhancement by forskolin; D-600 blocked potassium-induced prolactin release. Ionomycin had no effect on basal cAMP synthesis by tumor cells and inhibited slightly the forskolin-induced increase in nucleotide synthesis. The results suggest that cAMP acts, at a site distal to the entry of calcium into the cytosol, to enhance the amount of prolactin released in response to an increase in the cytosolic calcium concentration.

Prolactin release from the anterior pituitary gland is regulated by a variety of peptide hormones and releasing factors (1–4). However, the intracellular mechanisms underlying the control of prolactin release by these substances remain elusive. The classic belief is that the cytosolic calcium concentration controls the rate of hormone release (5), and this appears to be true for the release of prolactin (6, 7). The intracellular messenger, cAMP, also promotes hormone release (8). Cyclic AMP and calcium nearly always function in concert in controlling cellular function (9) and, consistent with this, cAMP enhances basal and evoked prolactin release (10–12), both calcium-dependent phenomena (6).

Our study was carried out to determine whether cAMP enhances calcium-evoked prolactin release by potentiating the entry of calcium into the cytosol. Because of the heterogeneous cellular composition of the anterior pituitary gland, it is virtually impossible to unambiguously associate biochemical variables common to all pituitary cells, such as the levels of calcium and cAMP in the cytosol, with the release of prolactin from a particular type of cell. Therefore, we have utilized the prolactin-secreting 7315c tumor cell as a convenient model of a normal mammatroph (13). The cytosolic calcium concentration of the tumor cells was raised with either ionomycin, a calcium ionophore (14), or a potassium challenge, a procedure believed to transiently open voltage-dependent calcium channels (5). The cytosolic calcium concentration in 7315c cells during these manipulations was measured using the Quin 2 fluorescent dye technique (15). Cyclic AMP synthesis was enhanced by forskolin, a drug which stimulates adenylate cyclase by bypassing physiological receptors on the cell surface (16). The results of our study indicate that cAMP does not enhance calcium-evoked IR-PRL release by potentiating the amount of calcium entering the cytosol. Rather, in the 7315c tumor cell, amplification of hormone release by cAMP occurs at a site distal to the entry of calcium.

Materials and Methods

Chemicals. Drugs and chemicals were obtained from the following sources: ionomycin, Quin 2/AM, and forskolin, Behring Diagnostics (La Jolla, CA); DMSO, 8Br-cAMP, 3-isobutyl-1-methylxanthine, Sigma Chemical Co. (St. Louis, MO); D-600, Knoll AG Chemische Fabriken (Ludwigshafen, West Germany); BSA (fraction V), Miles

ABBREVIATIONS: IR-PRL, immunoreactive prolactin; Quin 2, 2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-amino-quinoline; DMSO, dimethylsulfoxide; BSA, bovine serum albumin; EMEM, Eagle's minimal essential medium; HBSS, Hanks' balanced salts solution; EMEM/BSA, EMEM with 0.25% BSA; HBSS/BSA, HBSS with 0.02% BSA; 8Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

Laboratories, Inc. (Elkhart, IN); EMEM and HBSS, Grand Island Biological Co. (Grand Island, NY). HBSS was also prepared with chemicals from the J. T. Baker Co. (Phillipsburg, NJ). [^{125}I]Prolactin (rat; 50 μCi/μg) and [^{125}I]cAMP (2000 μCi/μg) were obtained from Meloy Laboratories, Inc. (Springfield, VA); and rat prolactin antiserum (anti-rPRL-S-8) and standard rat prolactin (AFP-4459B) were obtained from the National Hormone and Pituitary Program (Baltimore, MD).

Preparation of tumor cells for experiments. Rat 7315c tumor cells were grown *in vivo* and dispersed for use in experiments as previously described (13). In preparation for each experiment, dispersed cells were incubated in a volume of 50 ml at a concentration of 10^6 cells/ml of EMEM/BSA for 2 hr (37°) under a humidified atmosphere of 95% air and 5% CO_2.

Determination of IR-PRL release from 7315c tumor cells. Cells from two Petri dishes (100,000,000 cells) were gently dislodged and the cell suspension was centrifuged (5 min, 200 × *g*). The cell pellet was resuspended in HBSS/BSA (37°) to achieve a concentration of 4×10^6 cells/ml. The amount of IR-PRL in the incubation medium at zero time was determined as follows: 250 μl of the cell suspension was added to 750 μl of HBSS/BSA and immediately centrifuged for 30 sec in a Microfuge B (Beckman); aliquots of the supernatant were then stored on ice until the assay for IR-PRL was begun. Experimental incubations were begun immediately after the collection of these zero time samples. Each incubation contained six experimental conditions (three replicates per condition); two incubations, separated by no more than 15 min, were performed with the cells from each 100,000,000 cells; zero time samples were taken for each experimental incubation. Incubations for one experiment were completed within 2 hr.

The standard treatment protocol for the study of IR-PRL release was as follows. The 7315c cells were incubated for 5 min in the presence of either forskolin or its vehicle, DMSO; ionomycin was then added to the incubation medium or the concentration of potassium was elevated by adding HBSS/BSA in which sodium was replaced by potassium, and the incubation was continued for 5 min. Cells, drugs, and potassium were added with an automatic pipette (Eppendorf Repeater) in volumes of at least 250 μl to achieve mixing. Incubations were carried out at 37° in HBSS/BSA under a normal atmosphere and were stopped by centrifugation as described above. Additional details are presented in the legends to the figures.

Determination of IR-PRL release and cytosolic calcium concentration from Quin 2-loaded cells. Cells were resuspended in fresh EMEM/BSA at a concentration of 5,000,000 cells/ml. A 5-μl aliquot of a 10 mM solution of Quin 2/AM in DMSO was added to the cell suspension to achieve a final concentration of 5 μM. The cell suspension was incubated with gentle agitation for 15 min in a 37° water bath. The cells were then centrifuged at 200 × *g* for 5 min, resuspended in EMEM/BSA, and centrifuged again before the final resuspension in EMEM/BSA at 5×10^6 cells/ml. Aliquots (1 ml) were placed in plastic tubes and incubated for 5 min at 37° in a humidified atmosphere of 5% CO_2 in air. At the end of this period the tubes were capped and the cell aliquots were kept at room temperature until use. This procedure was found to minimize the leakage of Quin 2-free acid from the cells.

For fluorescence determinations, one aliquot of cells, previously exposed to Quin2/AM, was added to 10 ml of HBSS/BSA (25°) and centrifuged (200 × *g* for 5 min). The supernatant fluid was then removed, and the cells were washed twice by resuspension/centrifugation in 10 ml of the HBSS/BSA. This washing protocol removes fluorescent substances from the medium. The cells were finally resuspended in HBSS/BSA at a cell density of 10^6 cells/ml. Three ml of the cell suspension were then placed in a quartz fluorimeter cuvette in a temperature-controlled (37°) Perkin Elmer fluorescence spectrometer (model 3000) and stirred by a magnetic stirrer. The excitation wavelength was 339 nm (slit width = 5 nm) and the emission wavelength was 492 nm (slit width = 10 nm). At the beginning of each experiment, the formation of Quin 2-free acid was verified by identifying the characteristic emission peak of Quin 2-free acid at 492 nm. If this peak was not evident, the batch of cells was discarded.

The Quin 2-loaded cells were subjected to an incubation protocol similar to the one described above, i.e., a 5-min treatment of the cells with forskolin (10 μM) followed by the addition of ionomycin or potassium to the medium and a continuation of the incubation for 5 min. Ionomycin and forskolin were added from 1000-fold concentrated stock solutions (the solvents themselves were determined to have no effect on the fluorescence signal). The extracellular potassium concentration was increased by replacing half of the cell suspension with a balanced salt solution in which sodium had been replaced by potassium such that the final potassium concentration was 60 mM (the density of the cells was halved). The fluorescence signal was recorded during the 5-min preincubation with forskolin or vehicle alone and for 5 min after the addition of either ionomycin or high potassium. Aliquots (300 μl) of the cell suspension were taken just before (zero time) and 5 min after the challenge with either ionomycin or high potassium. These samples were centrifuged (10,000 × *g*, 5 sec) and stored on ice until the prolactin radioimmunoassay was done.

The fluorescence signals obtained in these experiments were calibrated as follows. At the end of an experiment, the cell suspension was sonicated using a Branson sonicator (10 W, 5 sec). The fluorescence signal from this sonicate corresponded to a calcium concentration of 1.5 mM. This signal was taken as maximal fluorescence (F_{max}). The minimal fluorescence signal (F_{min}) was obtained by the addition of sufficient EGTA to reduce the free calcium concentration to below 1 nM. These values of F_{max} and F_{min} were used to determine the free calcium concentration corresponding to the experimental fluorescence signals with the equation detailed by Tsien *et al.* (15). Any change in the autofluorescence after sonication of the cell suspension was estimated in unloaded cells and was substracted from the F_{max} values obtained in loaded cells. Autofluorescence was never more than 10% of F_{max}.

Determination of cAMP. A million cells were incubated (37°) for the indicated time periods in 1 ml of HBSS/BSA with the indicated concentrations of drugs. Incubations were stopped by placing the test tubes in a boiling water bath for 2 min. The tubes were then centrifuged for 10 min, and aliquots (500 μl) were stored frozen until the radioimmunoassay for cAMP was begun.

Radioimmunoassays. Radioimmunoassays for prolactin and cAMP were performed as described previously (13, 17). The amount of IR-PRL released by 10^6 cells was expressed as the amount present at the end of the 10-min protocol less the amount present at zero time.

Statistics. Each experiment was repeated at least three times, on different days. Drug effects and interactions were first estimated separately for each repetition: the effect of a drug was estimated by the response at the highest tested concentration minus the response in the absence of the tested drug; interactions between drugs were estimated by the difference between one drug's effect with and without the other drug present. Estimated effects and interactions were then averaged over all repetitions. Variability among repetitions was used to calculate standard errors for estimates. Effects and interactions were tested for statistical significance using two-sided, 0.05-level *t* tests.[1]

Results

Forskolin enhances ionomycin- and potassium-induced IR-PRL release. The basal rate of release of IR-PRL from the tumor cells was 6 ng/million cells/10 min (Fig. 1). Ionomycin (3 μM) or potassium (43.2 mM), added at the midpoint of a 10-min incubation, elicited the secretion of about 20 ng of IR-PRL/million cells above the basal release (Fig. 1). If the cells were exposed to forskolin for 5 min before and during

[1] We thank Dr. Richard Raubertas (Biometry and Field Studies Branch, National Institute of Neurological and Communicative Disorders and Stroke) for his advice and assistance with the statistics.

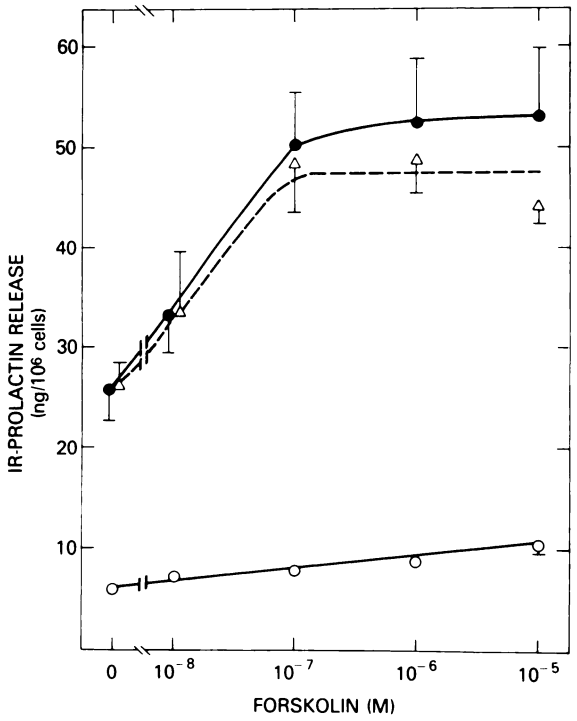

Fig. 1. Forskolin enhances ionomycin- and potassium-evoked IR-PRL release from 7315c cells. Aliquots (250 μl) containing 10^6 cells were added to 250 μl of HBSS/BSA containing forskolin (final concentration indicated on the *abscissa;* 0.1% DMSO was present in all samples), and these samples were incubated for 5 min at 37°. After 5 min, 500 μl of HBSS/BSA were added to some samples (○). Other samples received either 500 μl of HBSS/BSA containing ionomycin (●; final concentration, 3μM) or 500 μl of a high potassium HBSS/BSA (△; final concentration of potassium 43.2 mM). Incubations were continued for 5 min and then terminated by centrifugation. (The concentration of forskolin was decreased by 50% during the second 5 min of incubation.) The amount of IR-PRL released into the medium was determined by radioimmunoassay, as described in Materials and Methods. Results are expressed as the mean ± SE from three separate experiments. The stimulatory effects of forskolin, ionomycin, and potassium on IR-PRL release and the interaction between forskolin and either ionomycin or potassium in enhancing IR-PRL release were all statistically significant.

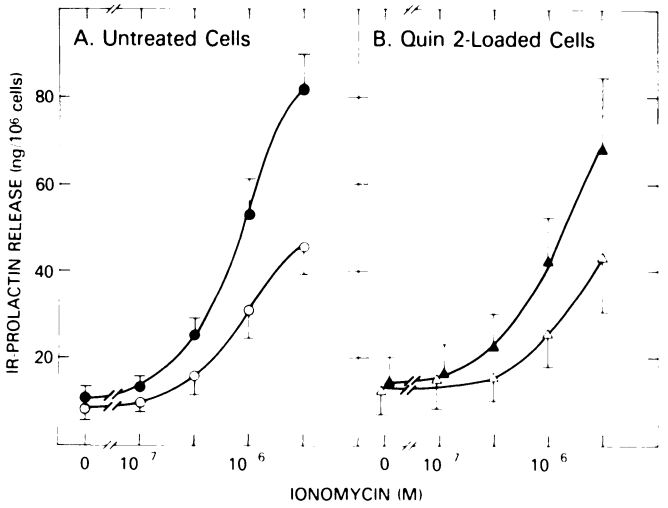

Fig. 2. Forskolin enhances evoked release of IR-PRL from either untreated or Quin 2-loaded 7315c tumor cells. The 7315c cells were loaded with Quin 2 by exposing them to Quin 2/AM as described in Materials and Methods (B). In the same experiment, the loading procedure was followed with another sample of tumor cells except that Quin 2/AM was omitted (A). After the loading procedure the cells were used immediately in an incubation protocol similar to that described for Fig. 1: 1) cells were pretreated for 5 min with either vehicle (○, △) or forskolin (10 μM; ●, ▲), 2) ionomycin was added to the incubation medium to give the indicated concentrations and the incubation was continued for 5 min, and 3) the incubation was terminated by centrifugation. The amount of IR-PRL released into the medium was determined by radioimmunoassay, as described in Materials and Methods. Results are expressed as the mean ± SE from three separate experiments. For both A and B, the stimulatory effect of ionomycin on IR-PRL release and the interaction between forskolin and ionomycin in enhancing prolactin release were each statistically significant. The effect of ionomycin and the interaction between forskolin and ionomycin were not significantly affected by Quin 2-loading.

the challenge with either ionomycin or potassium, the amount of IR-PRL released in response to either agent was enhanced. The maximal enhancement, which was the release of an additional 25 ng of IR-PRL/million cells, was observed with concentrations of forskolin between 0.1 nd 10 μM (Fig. 1). Forskolin enhanced ionomycin- and potassium-induced release with the same molar potency. Forskolin, alone, caused the release of only 4 ng of IR-PRL/million cells above basal during a 10 min incubation. The order of drug addition proved critical in that the synergy between forskolin and either ionomycin or potassium was only observed if the cells were treated with forskolin before ionomycin or potassium, and not vice versa (data not shown).

Forskolin did not alter the molar potency of ionomycin as a stimulant of IR-PRL release. Concentrations of ionomycin greater than 100 nM were necessary to increase IR-PRL release (Figs. 2A and 3A). At each effective concentration of ionomycin, cells pretreated with forskolin released more IR-PRL than did the vehicle-treated control cells.

8Br-cAMP (10 mM) also enhanced ionomycin-evoked IR-PRL release from 7315c cells when present 5 min prior to challenge with the ionophore; 8Br-cAMP had little effect, alone, on hormone release (Table 1).

The calcium channel antagonist, D-600 (7.5 μM), did not affect ionomycin-induced IR-PRL release or its enhancement by forskolin (data not shown). In contrast, D-600 (7.5 μM) blocked IR-PRL release induced by a maximally stimulatory concentration of potassium (43.2 mM; data not shown).

Forskolin does not alter ionomycin- or potassium-induced increases in cytosolic calcium concentrations. Loading 7315c cells with Quin 2 did not adversely affect the release of IR-PRL from these cells (Fig. 2). Neither the ionomycin-stimulated increase in IR-PRL release nor its enhancement by forskolin was significantly affected by introduction of Quin 2 into the cells. The concentration of calcium within the 7315c cells, as determined by the Quin 2 method, was approximately 100 nM. During a 10-min experimental incubation, forskolin had no effect upon the basal concentration of cytosolic calcium (Fig. 3B). Exposure of the cells to ionomycin increased the concentration of cytosolic calcium, in a concentration-dependent manner, by as much as 15-fold. Forskolin pretreatment had no effect upon the increased concentration of cytosolic calcium elicited by various concentrations of ionomycin (Fig. 3B). However, forskolin enhanced ionomycin-evoked hormone release from these cells (Fig. 3A). At each effective concentration of ionomycin, cells pretreated with forskolin released more IR-PRL than did vehicle-treated cells.

A challenge of Quin 2-loaded 7315c cells with 60 mM potassium raised the cytosolic calcium concentration from 100 nM

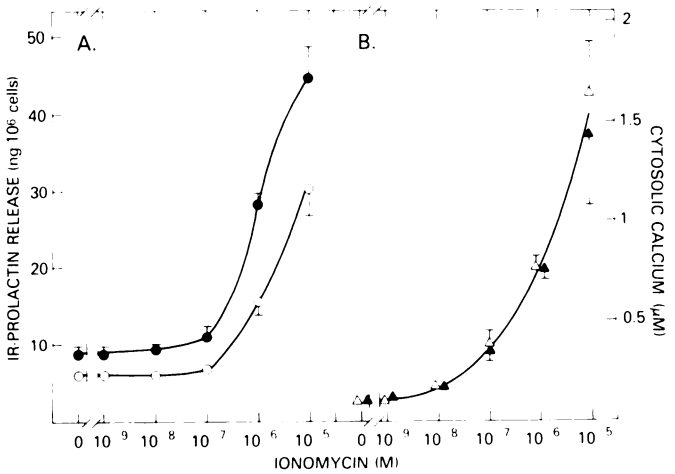

Fig. 3. Forskolin enhances ionomycin-evoked IR-PRL release but not the ionomycin-evoked increase in the cytosolic calcium concentration. Cells, loaded with Quin 2 as described in Materials and Methods, were transferred to a cuvette in a fluorimeter and incubated for 5 min at 37° in the presence of either 10 μM forskolin (●, ▲) or its vehicle (○, △). Ionomycin (final concentrations as indicated) was then added to the cuvette and the incubations were continued for 5 min. Fluorescence readings were taken throughout the incubation period. Results are expressed as the mean ± SE from 5 separate experiments. A. The amount of IR-PRL released into the medium during the 10-min incubations was determined by radioimmunoassay as described in Materials and Methods. B. The maximal concentration of cytosolic calcium attained following the addition of the indicated concentrations of ionomycin was determined from the fluorimeter tracing, as described in Materials and Methods. The stimulatory effects of forskolin and ionomycin on IR-PRL release and the interaction between the two drugs in enhancing IR-PRL release were all statistically significant. Ionomycin significantly increased the cytosolic calcium concentration. However, forskolin had no statistical effect on the cytosolic calcium concentration, nor was there any statistically significant interaction between forskolin and ionomycin on the cytosolic calcium concentration.

TABLE 1

8Br-cAMP enhances ionomycin-evoked IR-PRL release

Cells (10^6 in a volume of 500 μl of HBSS/BSA) were pretreated for 5 min at 37° with no drug, 8Br-cAMP, or forskolin. After 5 min, 500 μl of either HBSS/BSA or HBSS/BSA containing ionomycin (final concentration, 3 μM) was added. Incubations were continued for 5 min and then terminated by centrifugation. The amount of IR-PRL released into the medium was determined by radioimmunoassay, as described in Materials and Methods. Results were expressed as the mean ± SE from four separate experiments. The stimulatory effects of 8Br-cAMP and ionomycin and the interactions between either 8Br-cAMP or forskolin and ionomycin were all statistically significant.

Pretreatment	IR-PRL release	
	No ionomycin	Ionomycin (3 μM)
	ng/10⁶ cells	
None	3.9 ± 0.4	21.3 ± 5.0
8Br-cAMP (10 mM)	8.7 ± 1.1	40.8 ± 9.1
Forskolin (10 μM)	7.0 ± 1.6	51.4 ± 10.5

to 300 nM and, in the same cells, elicited a 3-fold increase in hormone release (Fig. 4). As was seen with ionomycin, forskolin had no effect on the basal cytosolic calcium concentration and very little effect on IR-PRL release. Pretreatment of the cells for 5 min with forskolin did not affect the potassium-evoked increase in cytosolic calcium concentration but did double (to a 6-fold increase) the potassium-stimulated increase in IR-PRL release.

Forskolin increases cAMP production by 7315c tumor cells. In accord with data from many other tissues, forskolin stimulated the production of cAMP by 7315c tumor cells.

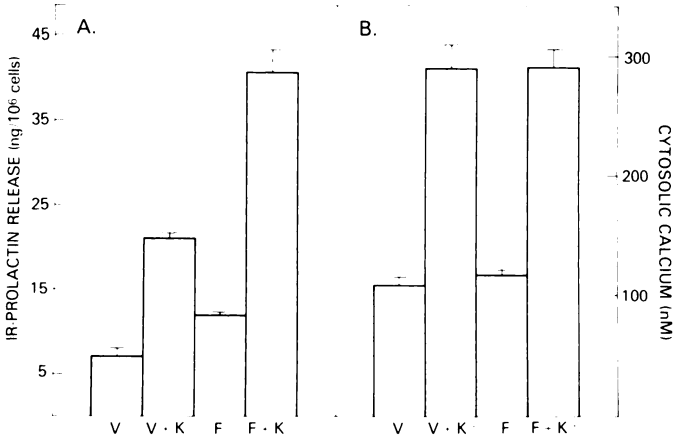

Fig. 4. Forskolin enhances potassium-evoked IR-PRL release but not potassium-evoked increase in cytosolic calcium concentration. Cells, loaded with Quin 2 as described in Materials and Methods, were transferred to a cuvette in a fluorimeter and incubated for 5 min at 37° in the presence of either 10 μM forskolin (*F*) or its vehicle (*V*). Then, half of the cell suspension in the cuvette was replaced with either normal balanced salt solution or a balanced salt solution with an elevated potassium concentration (K^+) to give a final concentration of 60 mM potassium, and the incubation was continued for 5 min. The results are expressed as the mean ± SE from three separate experiments. A. The amount of IR-PRL released into the medium during the 10-min incubations was determined by radioimmunoassay as described in Materials and Methods. B. The maximal concentration of cytosolic calcium attained following the addition of potassium was determined from the fluorimeter tracing, as described in Materials and Methods. The stimulatory effects of forskolin and potassium on IR-PRL release and the synergistic interaction between the two drugs were all statistically significant. Potassium significantly increased the cytosolic calcium concentration. However, forskolin had no statistically significant effect on the cytosolic calcium concentration, nor was there any statistically significant interaction between forskolin and potassium on the cytosolic calcium concentration.

Exposure of cells to forskolin (10 μM) increased the level of cAMP 17-fold within 15 sec (Fig. 5). Ionomycin did not affect the basal level of cAMP but caused a slight decrease in the amount of cAMP synthesized in response to forskolin. Qualitatively similar results were obtained when 1 mM 3-isobutyl-1-methylxanthine was present during incubations.

Discussion

Forskolin increased the amount of IR-PRL released in response to ionomycin or a potassium challenge, while having little effect, itself, on hormone release during the short incubations used in these experiments. 8Br-cAMP mimicked forskolin in these respects, implying that the enhancement of release by forskolin is probably mediated by cAMP. The relative ineffectiveness of either forskolin or 8Br-cAMP, alone, during a 10-min incubation, upon IR-PRL release from 7315c cells proved beneficial in that it allowed a study of the effect of cAMP on stimulated release without the complicating influence of a large effect on basal release. Although cAMP-mediated potentiation of calcium-evoked secretion has been reported in a variety of tissues (12, 18–24), the mechanisms underlying this phenomenon remain unclear and controversial.

The synergy between forskolin and ionomycin on IR-PRL release cannot be attributed simply to an enhanced cAMP production. The cellular content of cAMP was increased by forskolin, but ionomycin had no effect on basal cAMP levels, and it inhibited the forskolin-induced increase in cAMP.

The synergy between forskolin and ionomycin on IR-PRL

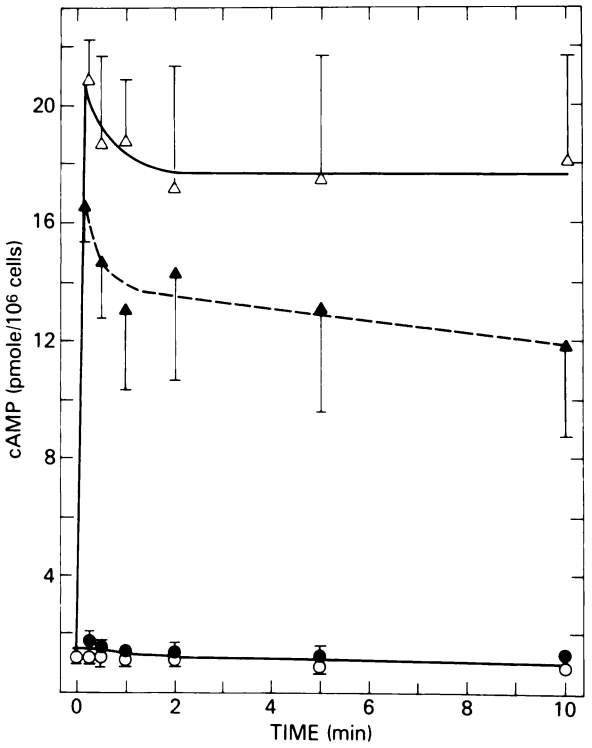

Fig. 5. Effects of forskolin and ionomycin on cAMP production by 7315c cells. One-ml aliquots of 7315c cells (10^6 cells/ml) were incubated for the indicated periods of time in HBSS/BSA at 37°. Cells were incubated in the absence of drugs (○) or in the presence of 3 μM ionomycin (●), 10 μM forskolin (△), or the combination of 3 μM ionomycin and 10 μM forskolin (▲). Incubations were stopped by transferring the aliquots to a boiling hot water bath. cAMP was determined by radioimmunoassay as described in Materials and Methods. The results are expressed as the mean ± SE from three separate experiments. Data for all times were pooled. Forskolin significantly stimulated cAMP production. Ionomycin, alone, had no significant effect on cAMP production, but it significantly inhibited the effect of forskolin.

release was also not due to enhanced entry of calcium into the cytosol. Using the Quin 2 method, we demonstrated that forskolin did not change the basal cytosolic calcium concentration or the cytosolic calcium concentration following treatment with ionomycin. Consistent with the results of the Quin 2 study was the observation that forskolin did not shift to the left the dose-response curve for the stimulation of IR-PRL release by ionomycin. If forskolin were potentiating ionomycin-evoked increases in the cytosolic calcium concentration, then ionomycin would be more potent in stimulating IR-PRL release in its presence. Forskolin also had no effect on the cytosolic calcium concentration induced by a potassium challenge.

In some cells, cAMP facilitates depolarization-evoked calcium entry by acting at voltage-dependent calcium channels in the cell membrane (25–28). Cyclic AMP may be increasing calcium entry through voltage-dependent channels in the 7315c cell. (The Quin 2 method may not be sensitive enough to detect a localized increase in the calcium concentration near the cell membrane.) However, enhanced calcium entry through voltage-dependent channels is neither necessary nor responsible for the synergy between ionomycin and forskolin upon IR-PRL release from 7315c cells (29). The calcium channel antagonist, D-600, had no effect on ionomycin-induced IR-PRL release or its enhancement by forskolin, which implies that forskolin was not acting at voltage-dependent channels. The efficacy of D-600 at voltage-dependent calcium channels was demonstrated by the fact that D-600 blocked IR-PRL release evoked by a potassium challenge.

To account for the cAMP-mediated potentiation of both ionomycin- and potassium-evoked IR-PRL release, we hypothesize that the effect of cAMP is at a site distal to the entry of calcium into the cytosol. Although the precise nature of the change induced by cAMP is unknown, it is clear that cAMP is increasing the capacity of the pituitary tumor cell to release IR-PRL in response to an increase in the cytosolic calcium concentration. We speculate that the preincubation period with forskolin or 8Br-cAMP allows a buildup of product at a rate-limiting step in the calcium-dependent release pathway or an alteration in the prolactin storage pattern making more prolactin available for release (9, 30).

Cyclic AMP sometimes does increase the cytosolic calcium concentration (31, 32). However, others have concluded, based on indirect evidence, that cAMP does not increase the cytosolic calcium concentration in bringing about the release of stored substances (20, 22). The present report is the first to directly demonstrate this using the Quin 2 method. The ability to enhance secretion without increasing the intracellular concentration of calcium is not unique to cAMP. Diacylglycerol, acting on protein kinase C, can stimulate exocytosis without increasing the cytosolic calcium concentration (33). The classic theory of Douglas is that the rate of secretion is regulated by the cytosolic calcium concentration. Our data and the data of others indicate that it is possible to increase the rate of hormone release without increasing the cytosolic calcium concentration.

References

1. Ojeda, S. R., A. Naor, and A. Negro-Vilar. The role of prostaglandins in the control of gonadotropin and prolactin secretion. *Prostaglandins Med.* **4**:249–275 (1979).
2. Tixier-Vidal, A., and D. Gourdji. Mechanism of action of synthetic hypothalamic peptides on anterior pituitary cells. *Physiol. Rev.* **61**:974–1011 (1981).
3. Meites, J., and W. E. Sonntag. Hypothalamic hypophysiotropic hormones and neurotransmitter regulation: current views. *Annu. Rev. Pharmacol. Toxicol.* **21**:295–322 (1981).
4. Leong, D. A., L. S. Frawley, and J. D. Neill. Neuroendocrine control of prolactin secretion. *Annu. Rev. Physiol.* **45**:109–127 (1983).
5. Douglas W. W. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmacol.* **34**: 451–474 (1968).
6. Tashjian, A. H., Jr., M. E. Lomedico, and D. Maina. Role of calcium in the thyrotropin-releasing hormone-stimulated release of prolactin from pituitary cells in culture. *Biochem. Biophys. Res. Commun.* **81**:798–806 (1978).
7. Albert, P. R., and A. H. Tashjian, Jr. Thyrotropin-releasing hormone-induced spike and plateau in cytosolic free calcium concentration in pituitary cells. *J. Biol. Chem.* **259**:5827–5832 (1984).
8. Labrie, F., P. Borgeat, J. Drouin, M. Beaulieu, L. Lagace, L. Ferland, and V. Raymond. Mechanism of action of hypothalamic hormones on the adenyhypophysis. *Annu. Rev. Physiol.* **41**:555–569 (1979).
9. Rasmussen, H., and P. Q. Barret. Calcium messenger system: an integrated view. *Physiol. Rev.* **64**:938–984 (1984).
10. Robberecht, P., M. Deschodt-Lanckman, J. C. Camus, P. De Neef, M. Lambert, and J. Christophe. VIP activation of rat anterior pituitary adenylate cyclase. *FEBS Lett.* **103**:229–233 (1979).
11. Tam, S. W., and P. S. Dannies. The role of adenosine 3′,5′ monophosphate in dopaminergic inhibition of prolactin release in anterior pituitary cells. Endocrinology **109**:403–408 (1981).
12. Delbeke, D., I. Kojima, P. S. Dannies, and H. Rasmussen. Synergistic stimulation of prolactin release by phorbol ester, A23187, and forskolin. *Biochem. Biophys. Res. Commun.* **123**:735–741 (1984).
13. Frey, E. A., and J. W. Kebabian. A mu-opiate receptor in 7315c tumor tissue mediates inhibition of immunoreactive prolactin release and adenylate cyclase activity. *Endocrinology* **115**:1797–1804 (1984).
14. Liu, C., and T. E. Hermann. Characterization of ionomycin as a calcium ionophore. *J. Biol. Chem.* **253**:5892–5894 (1978).
15. Tsien, R. Y., T. Pozzan, and T. J. Rink. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped, fluorescent indicator. *J. Cell Biol.* **94**:325–334 (1982).

16. Seamon, K. B., and J. W. Daly. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. *J. Cyclic Nucleotide Res.* **7**:201–204 (1981).
17. Harper, J. F., and G. Brooker. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after 2′ *O* acetylation by acetic anhydride in aqueous solution. *J. Cyclic Nucleotide Res.* **1**:207–218 (1975).
18. Pelayo, F., M. L. Dubocovich, and S. Z. Langer. Possible role of cyclic nucleotide in regulation of noradrenaline release from rat pineal through presynaptic adrenoceptor. *Nature (Lond.)* **274**:76–78 (1978).
19. Collen, M. J., V. E. Sutliff, G. Pan, and J. D. Gardner. Post-receptor modulation of action of VIP and secretion on pancreatic enzyme secretion by secretagogues that mobilize cellular calcium. *Am. J. Physiol.* **242**:G423–G428 (1982).
20. Rabe, C. S., J. Schneider, and R. McGee, Jr. Enhancement of depolarization-dependent neurosecretion from PC12 cells by forskolin-induced elevation of cyclic AMP. *J. Cyclic Nucleotide Res.* **8**:371–384 (1982).
21. Tsuruta, K., C. W. Grewe, T. E. Cote, R. L. Eskay, and J. W. Kebabian. Coordinated action of calcium ion and adenosine 3′,5′-monophosphate upon the release of alpha-melanocyte-stimulating hormone from the intermediate lobe of the rat pituitary gland. Endocrinology **110**:1133–1140, (1982).
22. Heisler, S. Forskolin potentiates calcium-dependent amylase secretion from rat pancreatic acinar cells. *Can. J. Physiol. Pharmacol.* **61**:1168–1176 (1983).
23. Henquin, J. C., and H. P. Meissner. The ionic, electrical and secretory effects of endogenous cyclic adenosine monophosphate in mouse pancreatic beta cells: studies with forskolin. *Endocrinology* **115**:1125–1134 (1984).
24. Malaisse, W. J., P. Garcia-Morales, S. P. Dufrane, A. Sener, and I. Valverde. Forskolin-induced activation of adenylate cyclase, cyclic adenosine monophosphate production and insulin release in rat pancreatic islets. *Endocrinology* **115**:2015–2020 (1984).
25. Cachelin, A. B., J. E. De Peyer, S. Kokubun, and H. Reuter. Calcium channel modification by 8-bromocyclic AMP in cultured heart cells. *Nature (Lond.)* **304**:462–464 (1983).
26. Henquin, J. C., W. Schmeer, and H. P. Meissner. Forskolin, an activator of adenylate cyclase, increases calcium-dependent electrical activity induced by glucose in mouse pancreatic beta-cells. *Endocrinology* **112**:2218–2220 (1983).
27. Reuter, H. Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature (Lond.)* **301**:569–574 (1983).
28. Bean, B. P., M. C. Nowycky, and R. W. Tsien. Beta-adrenergic modulation of calcium channels in frog ventricular heart cells. *Nature (Lond.)* **307**:371–375 (1984).
29. Bolger, G. T., C. R. Triggle, and D. J. Triggle. The action of the ionophore ionomycin in guinea-pig intestinal smooth muscle. *Can. J. Physiol. Pharmacol.* **61**:535–538 (1983).
30. Dannies, P. S. Prolactin: multiple intracellular processing routes plus several potential mechanisms for regulation. *Biochem. Pharmacol.* **31**:2845–2849 (1982).
31. Cocks, T. M., D. H. Jenkinson, and K. Koller. Interactions between receptors that increase cytosolic calcium and cyclic AMP in guinea-pig liver cells. *Br. J. Pharmacol.* **83**:281–291 (1984).
32. Morgan, N. G., R. Charest, P. F. Blackmore, and J. H. Exton. Potentiation of alpha-adrenergic responses in rat liver by a cAMP-dependent mechanism. *Proc. Natl. Acad. Sci. USA* **81**:4208–4212 (1984).
33. Baker, P. F. Multiple contols for secretion. *Nature (Lond.)* **310**:629–630 (1984).