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INHIBITION OF ATRIAL NATRIURETIC PEPTIDE SECRETION BY FORSKOLIN IN NONCONTRACTING CULTURED ATRIAL MYOCYTES

Hiroshi Iida[1] and Ernest Page[2,*]

[1]Department of Anatomy, Kyushu University, Fukuoka, Japan

[2]Department of Medicine - BH Box 137
The University of Chicago
5841 South Maryland Avenue
Chicago, Illinois 60637

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Secretory rates for immunoreactive atrial natriuretic peptide (ANP) by 7 - 8 day-old primary cultures of atrial myocytes from adult rats (with myocyte contraction inhibited by tetrodotoxin (TTX)) were (a) constant for at least two hours, and (b) significantly slowed by forskolin (1, 5, and 25 μM), dibutyryl cyclic adenosine monophosphate (1 mM), or isobutylmethylxanthine (100 μM). The substantial rates of ANP secretion which persisted in cells rendered noncontracting either by inhibiting Ca^{2+} influx via reduction of external [Ca^{2+}] to $<10^{-7}$ M or by inhibiting sarcoplasmic reticulum Ca^{2+} release with 100 μM ryanodine were significantly slowed by 25 μM forskolin, but forskolin sensitivity was lost by cells exposed simultaneously to external Ca^{2+} concentration of $<10^{-7}$ M and 100 μM ryanodine. Quiescent myocytes whose ANP secretory rate was depressed by forskolin remained responsive to secretory stimulation by phorbol ester.

Published data implicating phosphorylation via the cyclic AMP-mediated second messenger pathway in secretion of atrial natriuretic peptides (ANP) by intact mammalian atria show significant effects but are sometimes conflicting (1 - 3). Interpretation of these observations is complicated by the well-documented sensitivity of ANP secretion to passive stretch and to frequency and force of contractions (4 - 8). It would therefore be useful to examine the effects of varying intracellular cyclic AMP (cAMP) concentration on ANP secretion under baseline conditions characterized by absent or minimal passive stretch of the myocytes and absence of contractions.

Primary cultures of atrial myocytes from the hearts of neonatal rats (9 - 11) or adult rats (12) are well suited for such studies. For example, it can be shown that noncontracting primary cultures of atrial myocytes from adult rats maintain a significant "baseline" secretion even when external Ca^{2+} concentration is below 10^{-6} M and release of Ca^{2+} from the sarcoplasmic reticulum (SR) is inhibited with ryanodine (13); and that, under these base-

*To whom correspondence should be addressed.

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line conditions, activation of protein kinase C with a phorbol ester significantly increases the basal rate of ANP secretion apparently independently of Ca^{2+} influx or Ca^{2+} release by SR (13, 14).

In this paper, we have used the same cell culture system to investigate the effects of forskolin on ANP secretion of noncontracting atrial myocytes; to examine how the effects of forskolin are affected by the presence or absence of external Ca^{2+} and by inhibition of Ca^{2+} release from the SR by ryanodine; and to determine whether myocytes whose ANP secretory rate has been slowed with forskolin are still capable of being stimulated to more rapid secretion with an appropriate phorbol ester. Forskolin, a diterpene that increases cytoplasmic cAMP concentration and activates cAMP-dependent protein kinase, was chosen as the principal experimental tool because it bypasses both receptor binding and activation of G-protein, presumably by direct stimulation of adenylate cyclase (15).

METHODS

Primary cultures of atrial myocytes on 35 mm culture dishes were prepared from the hearts of mature, ether-anesthetized 300 - 350 g Sprague-Dawley rats as previously described (12). The myocytes were used for experiments on days 7 or 8 of culture, a time when many cells were contracting spontaneously at 37° C. The basic medium for growing the cultured cells was Gibco Medium 199 (M199) with Earle's salts supplemented with 10% fetal bovine serum (FBS). In addition to amino acids and other nutrients, this medium contains (in mM): K 5.4, Ca^{2+} 1.8, Mg 0.8, Cl 130, HCO_3 26, phosphate 1, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10 (pH 7.3).

The experimental design for measurement of ANP secretion was as previously described (12). All measurements of ANP secretion were made in protein-free medium (M199). We measured either the total concentration of immunoreactive ANP (in pmoles immunoreactive ANP/ml) accumulated in 2 ml of M199 in a culture dish, or the rate of ANP secretion sampled at appropriate intervals during 2 or 3 hours at 37° C (12). Before each experiment, the dishes were washed 4 - 5 times with serum-free M199. For rate measurements, three samples were taken from 3 dishes for each point in time and the ANP concentration of each sample was determined in duplicate. The means of these duplicate determinations were plotted as points, which were fitted to a straight line by the method of least squares.

The radioimmunoassay for ANP was as described previously (12).

Forskolin, 12-o-tetradecanoylphorbol-13-acetate (TPA), and 4α-phorbol-12,13-didecanoate (4α-PDD) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.025% or 0.031% for forskolin, and 0.006% for TPA and 4α-PDD.

Tetrodotoxin (TTX), TPA, 4α-PDD, 8-bromo-cyclic guanosine monophosphate (8Br-cGMP), dibutyryl cyclic adenosine monophosphate (db-cAMP), and isobutylmethylxanthine (IBMX) were obtained from Sigma Chemical Corp. (St. Louis, Missouri); forskolin from Calbiochem (San Diego, California); ryanodine from the Penick Co. (Lyndhurst, New Jersey); Medium 199 and FBS from Gibco (Grand Island, New York); anti-α human ANP antibody (RAS 8798) and rat or human atrial peptide (28 amino acids) from Peninsula Laboratories (Belmont, California); and ^{125}I-αANF from Amersham Corp. (Arlington Heights, Illinois).

Statistical analysis: Data on ANP secretion rates (Figures 1 - 3, 5) were fitted to lines by the method of least squares; for each experiment, the statistical significance of the differences between the slopes of the straight lines thus obtained were computed by an analysis of variance using the SAS program (SAS Institute, Inc., Cary, North Carolina). Differences between amounts of total ANP accumulated in 2 hours (Figure 4) were evaluated by a Student's t-test.

RESULTS AND DISCUSSION

We have previously shown that 10 μM TTX, which abolishes contractions, significantly decreases the rate of ANP secretion in cultured atrial myocytes (13). In all experiments to be described, all culture dishes sampled for comparison in a single figure were derived from the same cell suspension. Inspection of these TTX-treated cells under a phase contrast microscope at a magnification of 200X indicated that the cells were quiescent except for occasional rapid twitches involving only a small portion of a cell. Figures 1 - 3 show three experiments carried out in protein-free M199 designed to compare the rate of ANP accumulation of controls rendered quiescent by TTX with the rate for similarly TTX-treated myocytes exposed to 25 μM forskolin (Figure 1), 1 mM db-cAMP (Figure 2), or 100 μM IBMX (Figure 3). This experiment demonstrates that forskolin, db-cAMP, and IBMX all significantly reduced the rates of ANP accumulation ($P = .001$, .001, and .001 for the differences between experimental and control slopes of Figures 1, 2, and 3, respectively). None of these agents stimulated contraction of the cultured cells under the experimental conditions. Both the control and reduced rates of secretion remained reasonably constant during the two- or three-hour experiment. The data were therefore fitted to straight lines whose slopes and intercepts on the ordinate are given by the equations in the legend for each figure.

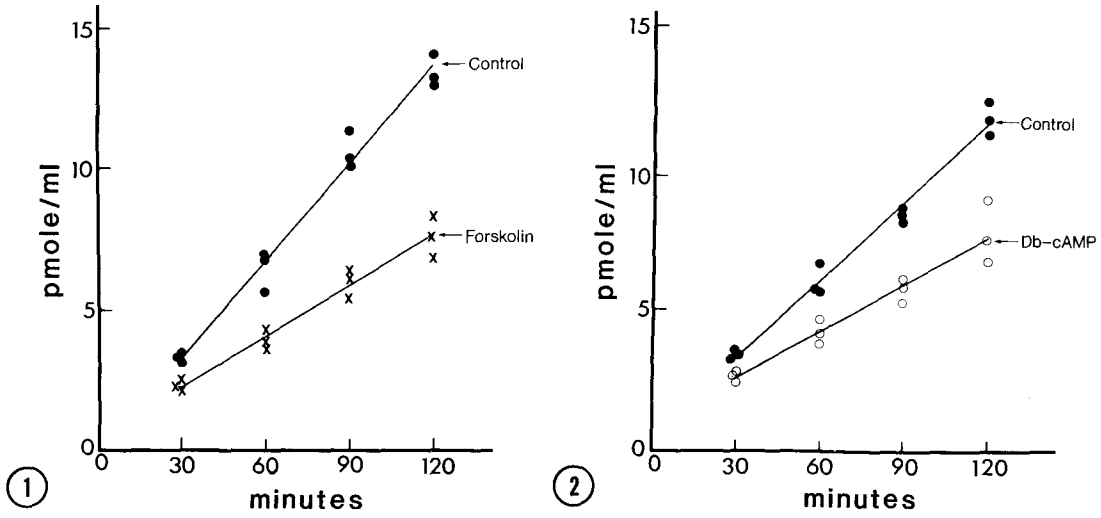

Figure 1 Effect of 25 μM forskolin on rate of ANP accumulation in protein-free medium containing 10 μM TTX. Least squares fits to lines yielded, respectively, for the control: $y = 0.12x - 0.21$ ($r^2 = .975$); forskolin: $y = 0.06x + 0.46$ ($r^2 = .957$).

Figure 2 Effect of 1 mM db-cAMP on rate of ANP accumulation in protein-free medium containing 10 μM TTX. Least squares fits to lines yielded, respectively, for the control: $y = 0.092x + 0.46$ ($r^2 = 0.979$); db-cAMP: $y = 0.056x + 0.87$ ($r^2 = 0.921$).

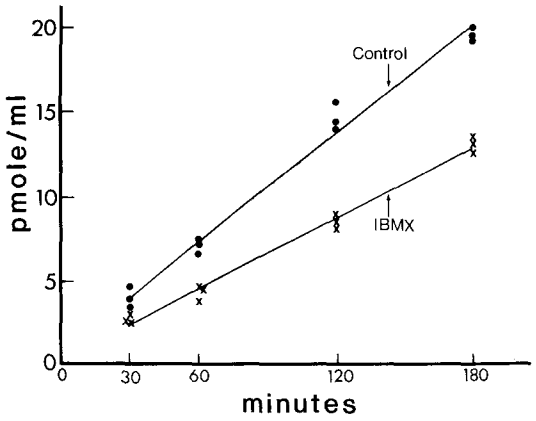

Figure 3 Effect of 100 μM IBMX on rate of ANP accumulation in protein-free medium containing 10 μM TTX. Least squares fits to lines yielded, respectively, for the control: $y = 0.11x + 0.96$ ($r^2 = .985$); IBMX: $y = 0.069x + 0.39$ ($r^2 = 0.989$).

To examine the dependence of the forskolin-induced slowing of ANP secretion on the forskolin concentration, we measured the total concentration of ANP accumulated after 2 hours of incubation in protein-free medium containing, respectively, forskolin concentrations (in μM) of 0, 1.0, 5.0, and 25. The ANP concentrations corresponding to these forskolin concentrations were, respectively (mean ± S.E. in pmoles/ml) 11.4 ± 0.5, 8.7 ± 0.2, 8.14 ± 0.09, and 7.6 ± 0.2. Figure 1 indicates that the slopes of the lines for steady-state secretion between 30 and 120 minutes have an approximately constant slope and near-zero extrapolated intercepts on the ordinate; the values of the ANP concentration at 2 hours are therefore scaled approximately as the steady-state rates of ANP accumulation. Since 25 μM forskolin, which yielded a large forskolin effect, produced no discontinuity or other unexpected effects on ANP secretion when the concentration of forskolin was raised to 25 μM, we felt justified in using this relatively high concentration for the subsequent studies of the effects of various manipulations on forskolin sensitivity described below.

Table 1 summarizes the results of an experiment in FBS-free modified M199 designed to compare the effects of three critical modifications on the sensitivity of noncontracting myocytes to suppression of ANP secretion by 25 μM forskolin. The modifications were (a) 100 μM ryanodine, (b) chelation of external Ca^{2+} with 4 mM EGTA, and (c) 100 μM ryanodine + 4 mM EGTA. Under these three conditions, contractions completely ceased. The quantity measured for each protocol was the amount of ANP accumulated in 2 ml of medium during 2 hours in presence or absence of forskolin. Table 1 shows that forskolin sensitivity was lost or became very small when Ca^{2+} influx and Ca release from SR were simultaneously inhibited by lowering external Ca^{2+} and exposure to ryanodine; whereas forskolin sensitivity was retained if Ca^{2+}

TABLE 1

Effects of Ryanodine and Reduced Extracellular Ca^{2+} on Forskolin-Mediated Reduction in ANP Accumulation

Inhibitor	Forskolin* concentration (μM)	ANP accumulated in 2 hours (pmoles/ml) (mean ± S.E., n = 3)	% reduction of ANP accumulation due to forskolin
100 μM ryanodine	0	15.7 ± 0.3	-32.3
	25	10.6 ± 0.1	
4 mM EGTA	0	9.3 ± 0.4	-20.3
	25	7.4 ± 0.4	
100 μM ryanodine + 4 mM EGTA	0	8.1 ± 0.6	-7.6
	25	7.4 ± 0.4	

* All solutions contained 0.025% DMSO

influx or release of Ca^{2+} from SR were separately (but not simultaneously) prevented.

Figure 4 illustrates an experiment to determine whether phorbol esters can stimulate ANP secretion by quiescent (TTX-treated) cells whose secretory rate has been depressed by forskolin. For this purpose the cultured cells were pre-incubated for 2 hours with 25 μM forskolin and 10 μM TTX. They were then washed and incubated for 40 additional minutes with forskolin + TTX containing one of the following: the 0.031% DMSO solvent only, 100 nM 4α-PDD (an inactive phorbol ester), or 100 nM TPA (a phorbol ester that stimulates protein kinase C). Figure 4 shows that TPA can significantly increase ANP accumulation by myocytes pretreated with forskolin ($P = .003$), while 4α-PDD is without effect. Thus treatment with forskolin for 2 hours did not interfere with the ability of the cultured myocytes to respond to stimulation by TPA.

Figure 5 compares the rate of ANP accumulation in the medium in non-contracting cells incubated in protein-free M199 in the presence of 10 μM TTX with that of cells incubated in an otherwise identical solution containing 1.0 mM 8Br-cGMP. The figure suggests that 8Br-cGMP, like forskolin, decreases the rate of ANP secretion, but the scatter of the data was such that the difference in slopes failed to achieve statistical significance ($P = 0.75$ for the slopes).

Our data show that forskolin slows the rate of ANP secretion in non-contracting myocytes. The effects of forskolin on secretion appear to be mediated by an elevation in cellular cAMP, since the secretion rate was

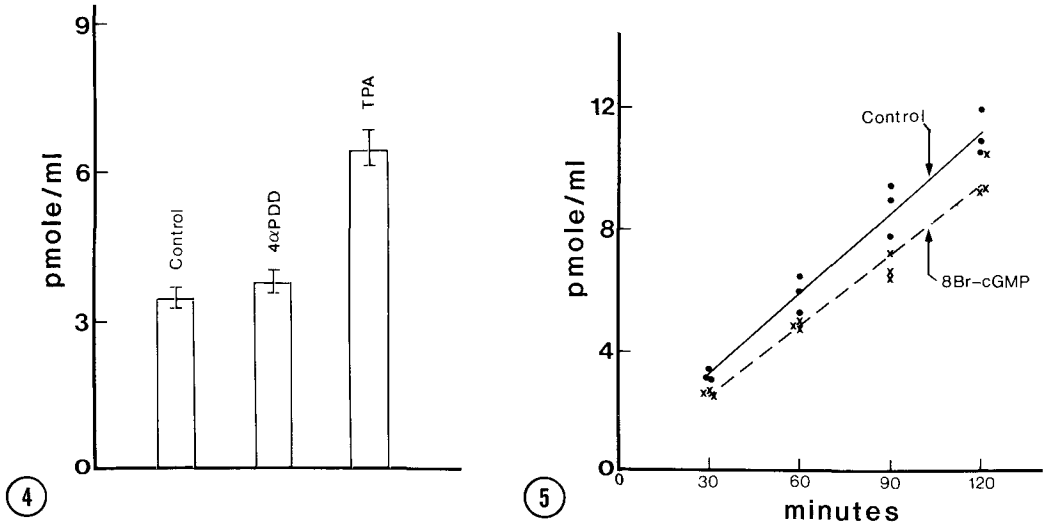

Figure 4 Effect of stimulating protein kinase C with the phorbol ester TPA on ANP secretion of atrial myocytes whose secretory rate was depressed by 2 hours of pre-incubation with 25 μM forskolin. The protein-free medium contained 10 μM TTX + 25 μM forskolin with or without the indicated phorbol ester. Left: control without phorbol ester; Middle: control containing 100 nM of the inactive phorbol ester 4α-PDD; Right: secretion stimulated with 100 nM TPA.

Figure 5 Effect of 1 mM 8Br-cGMP on rate of ANP accumulation in protein-free medium containing 10 μM TTX. Least squares fits to lines yielded, respectively, for the control: $y = 0.089x + 0.48$ ($r^2 = 0.965$); 8BR-cGMP: $y = 0.077x + 0.21$ ($r^2 = 0.972$).

similarly suppressed by db-cAMP and by an inhibitor of the phosphodiesterase which degrades cAMP. The results shown in Table 1 indicate that the sensitivity to forskolin is lost (or nearly so) if both transplasmalemmal influx and Ca^{2+} release from the SR are simultaneously prevented, but not when only one of these processes is inhibited. Further experiments are necessary to determine whether and to what extent cAMP-induced Ca^{2+} uptake by SR (16, 17) contributes to the suppressive effect of forskolin on ANP secretion. The effect of forskolin on ANP secretion of noncontracting atrial myocyte cultures differs from the phorbol ester (TPA)-activated stimulation of ANP secretion, which persists in spite of simultaneous reduction in external Ca^{2+} concentration and inhibition of Ca^{2+} release (14).

Differences between our findings of a forskolin-induced depression of ANP secretion in noncontracting atrial myocytes and the finding of a small, forskolin-induced increase in secretory rate in intact contracting atria reported by Ruskoaho, *et al.* (2) may well be due to differences between primary cultures and intact tissues. However, in view of the observation that forskolin stimulates voltage-sensitive Ca^{2+} current in isolated frog ventricular myocytes (18) and increases heart rate and force of contractions (2), it seems more probable that the difference reflects fundamental dissimilarities in the stimulus to secretion experienced by stretched, con-

tracting atria and unstretched quiescent atria. In this connection, it may be relevant that passive distension of noncontracting intact rat atria produces a several-fold increase in ANP secretory rate even when external Ca^{2+} is reduced to 0.02 mM and release of Ca^{2+} from the SR is inhibited with ryanodine (E. Page, unpublished observation).

The observation (Figure 5) that 8Br-cGMP slows the rate of ANP secretion in noncontracting cultured atrial myocytes is consistent with a similar finding in atria of intact, contracting rat hearts (2). Our data suggest that contractions are not required for the suppressive effect of this cGMP analogue.

ACKNOWLEDGMENTS

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REFERENCES

1. Schiebinger, R. J. (1985) Circulation 72 (Suppl. III): 310.
2. Ruskoaho, H., Toth, M., Ganten, D., Unger, T., and Lang, R. E. (1986) Biochem. Biophys. Res. Commun. 139: 266-274.
3. Schiebinger, R. J., Baker, M. Z., and Linden, J. (1987) J. Clin. Invest. 80: 1687-1691.
4. Dietz, J. R. (1984) Am. J. Physiol. 247: R1093-R1096.
5. Lang, R. E., Tholken, H., Ganten, D., Luft, F. C., Ruskoaho, H., and Unger, T. (1985) Nature (London) 314: 264-266.
6. Ledsome, J. R., Wilson, N., Courneya, C. A., and Rankin, A. J. (1985) Can. J. Physiol. Pharmacol. 63: 739-742.
7. Schiebinger, R. J., and Linden, J. (1986) Am. J. Physiol. 251: H1095-H1099.
8. Schiebinger, R. J., and Linden, J. (1986) Circ. Res. 59: 105-109.
9 Bloch, K. D., Scott, J. A., Zisfein, J. B., Fallon, J. T., Margolies, M. M., Seidman, C. E., Matsueda, G. R., Homcy, C. J., Graham, R. M., and Seidman, J. G. (1985) Science 230: 1168-1171.
10. Bloch, K. D., Seidman, J. G., Naftilan, J. D., Fallon, J. T., and Seidman, C. E. (1986) Cell 47: 695-702.
11. Sylvestre, D. L., Zisfein, J. B., Graham, R. M., and Homcy, C. J (1986) Biochem. Biophys. Res. Commun. 140: 151-159.
12. Iida, H., Barron, W. M., and Page, E. (1988) Circ. Res. 62: 1159-1170.
13. Iida, H., and Page, E. (1988) Biophys. J. 53: 134a.
14. Iida, H., and Page, E. (1988) FASEB J. 2: A533.
15. Seamon, K. B., and Daly, J. W. (1981) J. Cyclic Nucleotides Res. 7: 201-224.
16. Tada, M., Ohmani, F., Yamada, M., and Abe, H. (1979) J. Biol. Chem. 254: 319-326.
17. Tada, M., Kirchberger, M. A., Iolio, J. M., and Katz, A. M. (1975) Circ. Res. 36: 8-17.
18. Hartzell, H. C., and Fischmeister, R. (1987) Molec. Pharmacol. 32: 639-645.