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THE PHORBOL ESTER INDUCED ATRIAL NATRIURETIC PEPTIDE SECRETION IS STIMULATED BY FORSKOLIN AND BAY K8644 AND INHIBITED BY 8-BROMO-CYCLICGMP

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The role of intracellular signals in the regulation of atrial natriuretic peptide (ANP) release was studied using the isolated perfused rat heart. The phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), known to activate the protein kinase C pathway, produced a dose-dependent increase in perfusate ANP immunoreactivity. Bay k8644, a putative calcium channel activator, and forskolin, which stimulates adenylate cyclase, induced a sustained increase in ANP secretory rate. TPA in combination with either Bay k8644 or forskolin induced higher ANP secretion than the calculated additive value for each agent. 8-bromo-cyclicGMP and sodium nitroprusside, when given alone, had no effect on ANP secretion, but delayed the TPA-stimulated increase in perfusate ANP. ANP secretion appears therefore to be mediated both by the phosphoinositide and the cAMP system, whereas the cGMP pathway may be inhibitory.

Mammalian atrial myocytes synthetize and secrete a hormone called atrial natriuretic peptide (ANP), which causes natriuresis, diuresis and inhibition of smooth muscle contraction, aldosterone and renin release (1-4). Current evidence suggest that the major stimulus for ANP release is atrial stretch (5-8). Evidence that humoral factors and nerve activity directly influence ANP release is scanty. However, adrenaline may be one of those (9). There is also little information on what cellular mechanisms are involved in the mediation of ANP release.

Two major cellular signal pathways have been shown to promote hormone secretion from endocrine cells. One employs the second messenger cyclic adenosine monophosphate (cAMP), the other a combination of second mes-

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Abbreviations: ANP: atrial natriuretic peptide; TPA: 12-O-tetradecanoyl-phorbol-13-acetate; SNP: sodium nitroprusside; db-cAMP: dibuturyl-cyclicAMP; 8br-cGMP: 8-bromo-cyclicGMP.

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sengers that includes calcium ions, inositoltriphosphate and diacylglycerol (10-12). The rise in diacylglycerol concentration activates a calcium-activated, phospholipid-dependent protein kinase C and the increased cAMP activates protein kinase A, which by catalysing the phosphorylation of specific proteins appear to be involved in the mechanism of hormone secretion. The contribution of each pathway to hormone secretion can be assessed with pharmacological agents that mimic the action of a particular second messenger and therefore stimulate only one pathway. In previous work (9), we observed that the Ca^{2+} ionophore A23187 which introduce free calcium into the cell (13) and the phorbol esters which mimic the action of diacylglycerol by acting directly on C-kinase (14) both increased ANP, thereby suggesting that the calcium-activated protein kinase C may be involved in ANP secretion from atrial cardiocytes. Our present results, obtained from pharmacological studies in the isolated perfused rat heart, suggest that ANP secretion appears to be mediated both by the phosphoinositide and the cAMP system, whereas the cGMP pathway may be inhibitory.

Materials and Methods

All experiments of the present study were performed on the rat heart perfused according to the method of Langendorff as described previously (9). The hearts were perfused for one hour with Krebs-Henseleit buffer, pH 7.4, equilibrated with O_2/CO_2 (95:5), at 37°C with a constant flow of 5 ml/min to stabilize hormone secretion rates. To assess the hemodynamic effects of infused substances, the perfusion pressure was recorded on a Grass polygraph via a cannula in the aortic cannula connected to a pressure transducer (Statham P23BB). Isometric force of contraction was recorded by a strain gauge transducer (HP model FTA 1010) connected to the Grass polygraph. Heart rate was counted from contractions by rate mater (Ebru ratemeter EM100I). The hearts were submitted to a resting tension of 2 g. The basal values for heart rate and perfusion pressure prior infusion of substances were 242±3 beats/min and 36±1 mm Hg (n=74).

The ANP immunoreactive material (ANPir) in the perfusate was measured by radioimmunoassay (9). The radioimmunoassay was performed in 0.1 M tris buffer, pH 7.4 containing 0.1 % gelatin. Synthetic atriopeptin III (AP III) 1.95 pg-1000 pg/tube was used for to construct standard curves. The incubation mixture consisted of 0.15 ml buffer, 0.15 ml standard or perfusate, 0.1 ml antibody (final dilution 1:30.000), and 0.1 ml tracer (3500 c.p.m.). After an incubation of 48 hrs at 4°C separation of free from antibody-bound ^{125}I-peptide was achieved by adding 0.2 ml dextran-coated charcoal. Using this procedure, the lowest concentration of AP III yielding a binding different from that in the

absence of standard at the 95% confidence interval was 2 pg/tube. The 50% intercept was at 41 pg/tube. A complete cross-reaction was observed with AP I, APII and alpha-rat ANP. The serial dilutions of perfusate inhibited the binding of tracer to antibody in parallel with the standard curve of AP III.

Agonists were added into the coronary flow using an infusion rate of 0.5 ml/min for 30 min. The coronary venous effluents were collected at 2 min intervals, placed immediately on dry-ice, and stored at $-20^{o}C$ until assayed. DMSO, ethanol and Krebs-Henseleit buffer, which were used as vehicles, had no effect on ANPir release or hemodynamics at the concentrations used to dissolve the substances.

The phorbol ester, TPA, dibuturyl-cyclicAMP, 8-bromo-cyclicGMP and sodium nitroprusside were obtained from Sigma Chemicals, forskolin from Calbiochem and Bay K8644 from Bayer. TPA was dissolved in DMSO, Bay k8644 and forskolin in ethanol, all other in Krebs-Henseleit buffer. Final concentration of each solvent was less than 0.03%. The synthetic peptides atriopeptin I (AP I), AP II, APIII and alpha-rat ANP were obtained from Bachem AG (Bubendorff, Switzerland).

Results and Discussion

During perfusion of the rat heart at constant flow, administration of TPA into the perfusate produced a dose-dependent, slowly developing increase of ANPir in perfusate (Fig.1a). As reported previously, the biologically non-active phorbol ester, 4-α-phorbol-12,13-didecanoate did not alter the basal secretion of ANP, whereas the active phorbol-12,13-didecanoate induced a similar pattern of ANP release as obtained after TPA addition (9). These findings point to the possible involvement of C-kinase in the exocytotic secretion of ANP.

Since calcium and diacylglycerol act synergistically to activate C-kinase (10) the combination of phorbol ester and Bay k8644 which introduce free calcium into the cell (15) should result in a more than additive response. In the isolated perfused rat heart addition of Bay k8644 at a dose of $4x10^{-7}$ caused a sustained increase in contractile force and heart rate and transient increase in perfusion pressure. Bay k8644 alone only produced a 2-fold increase in basal ANPir secretion (Fig.1a). When given together with TPA, Bay k8644 produced a marked enhancement of TPA-induced ANPir secretion (Fig.1a, Table 1). This synergistic effect gives further support for a role of calcium-activated C-kinase in ANP secretion. Our results also suggest that ANP secretion is a Ca^{2+}-dependent process, which may be initiated by a rise in the

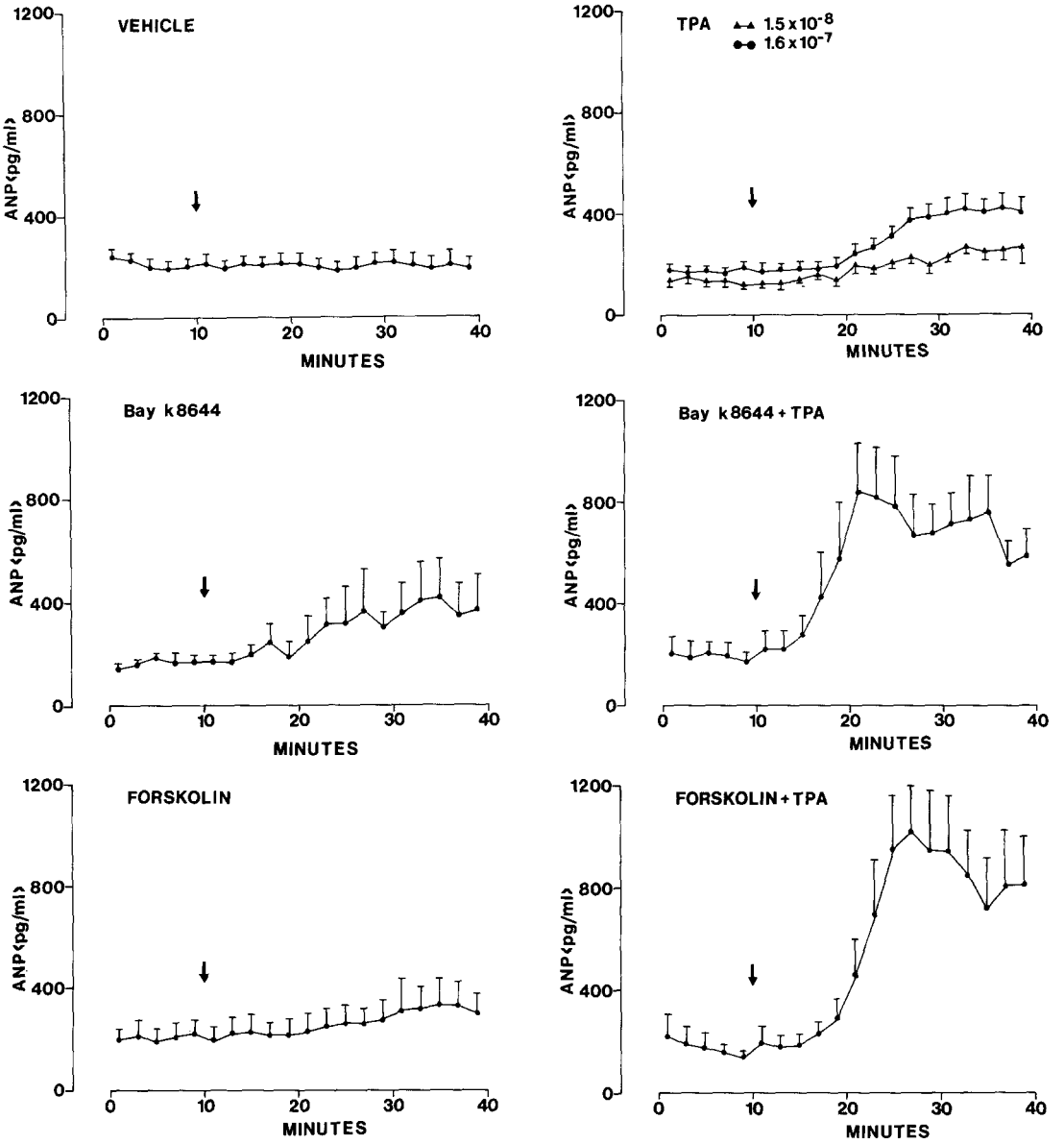

Figure 1a: Atrial natriuretic peptide (ANP) secretion into the perfusate from isolated perfused rat hearts. After a 10 min control period, agonists were added (arrows) into the perfusion fluid for 30 min. The coronary venous effluents were collected at 2 min intervals and assayed for ANP-like immunoreactivity by radioimmunoassay. Vehicle group consisted of 4 infusion of DMSO, ethanol and Krebs-Henseleit solution. The ANP secretion is expressed as pg/ml perfusate and each point is the mean value ± SEM from 5-12 separate experiments run on different isolated rat hearts. For doses see Table 1. TPA=12-O-tetradecanoyl- phorbol-13-acetate.

intracellular concentration of free Ca^{2+}. A similar observation has recently been made for a number of other hormones, such as, insulin, aldosterone, catecholamines and pituitary hormones (for reviews see 10-

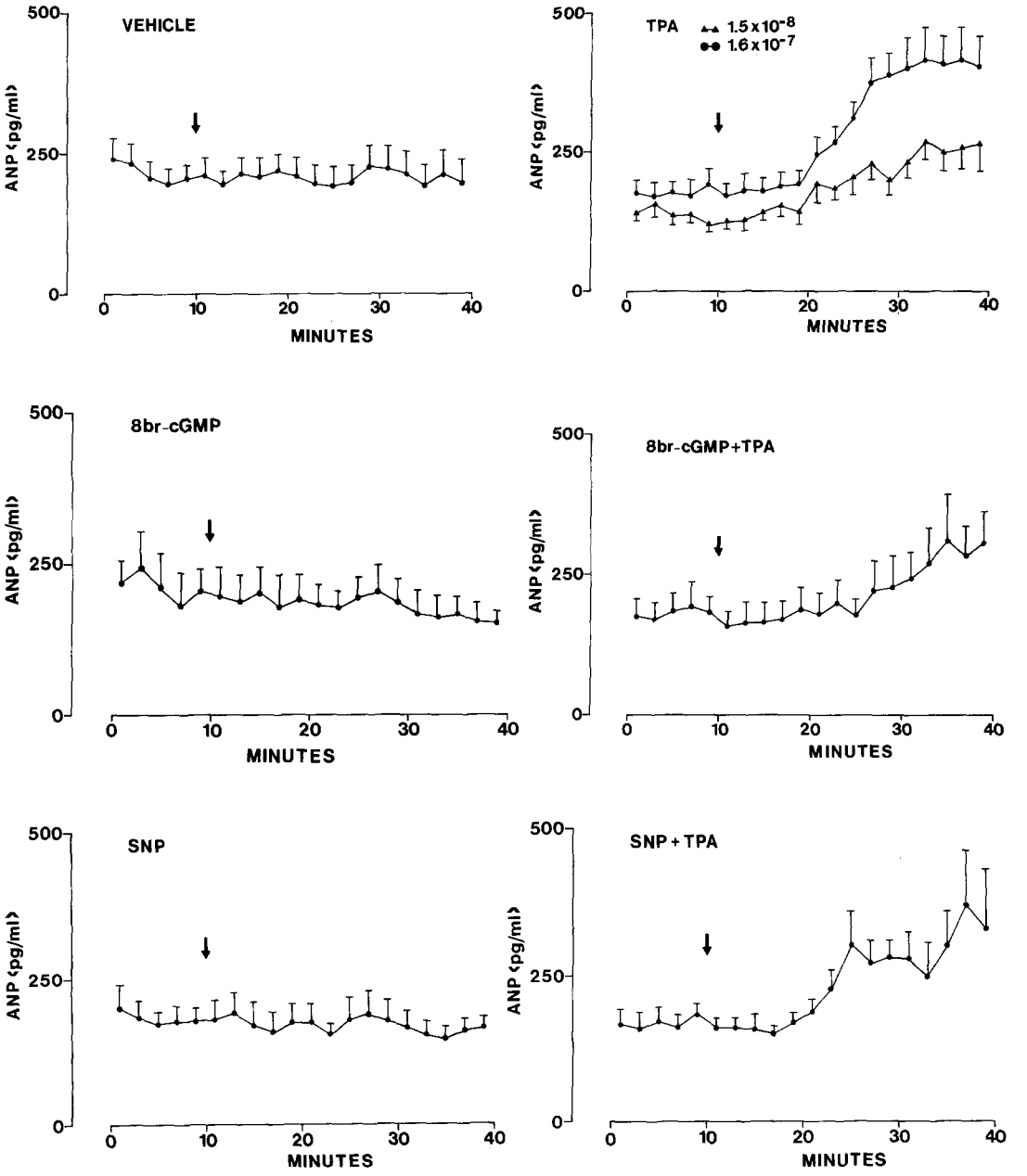

Figure 1b: Atrial natriuretic peptide (ANP) secretion into the perfusate from isolated perfused rat hearts. Hearts were perfused as described in the legend to Fig. 1 with the following exception: after 30 min perfusion of dibuturyl-cyclicAMP(db-cAMP), 8-bromo-cyclic GMP (8br-cGMP) or sodium nitroprusside (SNP) alone, the hearts were perfused for 30 min with the combination of TPA and db-cAMP, 8br-cGMP or SNP. TPA alone produced similar increase in ANPir secretion added to the perfusate during the first or second period. Both 8br-cGMP and SNP attenuated the TPA-induced increase in ANPir secretion; TPA alone increased secretion in 20 min from 198±27 to 402±53 (103%, n=12, $p<0.05$), the combination of TPA and 8br-cGMP from 182±30 to 245±57 (35%, n=5, ns.), and TPA and SNP from 169±19 to 279±44 (65%, n=6, ns.)(ANOVA followed by the Bonferroni t-test). Values are means±SEM for 5-12 separate experiments. For doses see Table 1.

Table 1. Stimulation of atrial natriuretic peptide (ANP) secretion by various agonists from isolated, perfused rat hearts

Group	n	dose	control ANPir (pg/ml)	max ANPir (pg/ml)	%
Vehicle	12		214 ± 26	203 ± 38	-9 ± 17
TPA	12	1.6×10^{-7}	198 ± 28	410 ± 53***	+143 ± 44
Bay k8644	5	4×10^{-7}	166 ± 24	379 ± 131	+109 ± 39
Bay K8644 + TPA	5		201 ± 52	669 ± 128***	+262 ± 4
Forskolin	5	1×10^{-6}	212 ± 48	323 ± 98	+46 ± 10
Forskolin + TPA	5		179 ± 48	828 ± 194***	+375 ± 76
db-cAMP	5	1.6×10^{-4}	216 ± 37	131 ± 13	-34 ± 9
db-cAMP + TPA	5		124 ± 16	326 ± 22**	+175 ± 24
8br-cGMP	5	1.3×10^{-4}	211 ± 46	189 ± 33	-1 ± 22
8br-cGMP + TPA	5		182 ± 30	284 ± 61	+79 ± 40
SNP	6	9×10^{-5}	182 ± 27	157 ± 21	-10 ± 9
SNP + TPA	6		169 ± 19	299 ± 65	+77 ± 35

The hearts were perfused as described in the legends to the Figs. 1a and 1b. The ANP immunoreactivity (ANPir) secretion is expressed as pg/ml perfusate and all result are expressed as means ± SEM. Control ANPir = mean of values prior to infusion. Max ANPir = mean of values during the last 10 min of infusion. For abbreviations see the legends to the Figs.1a and 1b. Statistical significance was ascertained by using the analysis of variance (ANOVA) followed by the Bonferroni t-test as approriate. *** indicates a significance level $p<0.001$, and ** indicates $p<0.01$.

12). Very recently, ANP produced in brain have been reported to release by membran depolarization and calcium-mediated mechanism (16,17).

To explore the possible physiological role of cyclic AMP in the ANP secretion, we infused a cyclic AMP analogue (dibuturyl-cyclicAMP, db-cAMP) and forskolin, a novel substance which activates adenylate cyclase and elevates cyclic AMP levels in myocardium (18). Forskolin alone increased heart rate and contractile force as described previously under these experimental conditions. Addition of forskolin (1×10^{-6}) into the perfusion fluid caused a small increase in ANPir secretion (Fig.1a), but when added together with TPA, the ANPir secreted was greater than the calculated additive value for these two agents (Table 1). Pretreatment with db-cAMP also enhanced the TPA-stimulated ANPir release (Table 1).

The results show that events in the calcium-activated protein C-kinase messenger system are intimately related to those in the cyclic AMP system in regulating ANP secretion from atrial cardiocytes.

The physiological function of cyclic GMP, as well as the mechanism by which this cyclic nucleotide is elevated, is still not clearly understood. It has been proposed that cyclic GMP may act as a negative, rather than a positive, messenger, providing an immediate feed-back control that prevents over-response (10,19). Our observation in the isolated perfused rat heart support this hypothesis, TPA-induced ANP secretion was delayed by treatment with 8-bromo-cyclic GMP and with sodium nitroprusside, which induces cyclic GMP formation (20)(Fig.1b and Table 1). The ANPir released in response to the various agents used in this study, was characterized by high pressure liquid chromatography, as previously described (5,9). In contrast to extracts of atrial tissue, which separate into several immunoreactive peaks, ANPir in the perfusate eluted as a single peak which eluted in the same position as synthetic ANP(1-28). This peptide has previously been shown to circulate in rat and human blood (1-4).

In conclusion, the present study is the first to suggest that there is a link between the components of the atrial natriuretic peptide system, the phosphoinositide system and the cyclic AMP system. The phorbol ester which mimic the action of diacylglycerol and calcium channel activator which increase the concentration of free intracellular calcium stimulate ANP release and when given together they potentiate each others effect. As reported for insulin and aldosterone (21,22) we observed that forskolin, which stimulates adenylate cyclase and elevates intracellular cAMP further enhanced the TPA induced ANPir release. In contrast, an increase in intracellular cyclic GMP may decrease ANP secretion.

Activation of the two second messenger pathways may be elicited by a number of endogenous agents binding to atrial receptors. These include

the neurotransmitters contained in cardiac nerves and circulating hormones. In addition, the actions of the electrical impulses originating regularly in the sinuatrial node and spreading through the atria, may also be mediated and modulated by these systems and contribute to ANPir release. In skeletal muscle inositol 1,4,5-triphosphate has recently been shown to act as a chemical second messenger between transverse-tubular membran depolarization and calcium release from sarcoplasmatic reticulum (23). The same may be true for the heart, where raised calcium levels, possibly together with calmodulin, could then promote hormone release. The contribution of cAMP to ANPir release may be to increase the number of calcium channels activated by depolarization and thereby to enhance calcium influx (24). Finally, it has been demonstrated that the cytosolic calcium concentration (25) and calcium sensitivity (26) depends on the length of the myocardial fibres. Whether or not the proteinkinases involved in this mechanism also mediate the phosphorylation of cellular proteins, which promote secretion of ANP from the atrial myocytes stimulated by atrial distension, remains to be clarified.

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