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Suppression of Programmed Cell Death by Intracellular cAMP Is not Mediated by Expression of Genes Encoding an Inhibitor of Apoptosis

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Abstract—The elevation of intracellular cAMP content is accompanied by expression of genes whose promoter contains a Ca^{2+}-cAMP responsive element. In vascular smooth muscle cells (VSMC), activation of cAMP signaling blocks apoptosis triggered by serum deprivation. In the present study we investigated the role of gene expression in the inhibition of apoptosis by cAMP. In VSMC transfected with E1A adenovirus, incubation in the absence of serum for 6 h led to 20-fold elevation of chromatin fragmentation and 10-fold activation of caspase-3 activity, these being employed as markers of apoptosis. Forskolin-induced activation of cAMP signaling was accompanied by 50% elevation of RNA synthesis and completely abolished the development of apoptosis during the initial 6 h incubation in growth factor-free medium. In 12 h apoptosis in forskolin-treated VSMC was slowly developed and after 24 h the content of chromatin fragments was 2-fold less than in control cells. Addition of actinomycin D and cycloheximide completely blocked RNA synthesis and decreased protein synthesis by 80%, respectively. Neither compound affected baseline apoptosis or its inhibition by forskolin. More than 70 newly phosphorylated proteins were observed by 2D-electrophoresis of VSMC after incubation with forskolin for 3 h; in 24 h the number of phosphoproteins triggered by forskolin was decreased by 2-3-fold. These results show that suppression of VSMC apoptosis under activation of cAMP signaling is mediated via posttranslational modification of pre-existing intermediates of the apoptotic machinery rather than by *de novo* synthesis of inhibitors of programmed cell death.

Key words: apoptosis, cAMP, RNA synthesis, protein synthesis

Initial stages of programmed cell death (apoptosis) possess tissue specific features that are lost at the terminal (universal) stages during activation of specific proteases (caspases). This is important for the development of pharmacological approaches for correction of diseases (e.g., Alzheimer's and Parkinson's diseases) characterized by premature cell death or accumulation of "unwanted" cells that appear during malignant conversion or tissue hypertrophy. Long-term maintenance of increased blood pressure and atherosclerosis are accompanied by vascular wall hypertrophy [1-4]. We earlier investigated the role of signal systems in the development of apoptosis of vascular smooth muscle cells (VSMC). We found that in contrast to immune system cells, VSMC are less sensitive to agonists of so-called death receptors (FAS-ligand, TNF-α); however, these cells undergo apoptosis in growth factor-free medium. Addition of the low-selectivity protein kinase inhibitor staurosporine and the highly selective inhibitors of serine/threonine phosphatases caliculin and okadaic acid also induced apoptosis of VSMC [5]. Activation of the cAMP signaling system [6] or increase of intracellular $[Na^+]/[K^+]$ ratio [7] attenuated the development of apoptosis of VSMC at the stage preceding caspase-3 activation. However, systems involved in protection of cells against apoptosis are tissue-specific. For example, Na^+/K^+-ATPase inhibitors exhibit the antiapoptotic effect in VSMC and neuronal cell culture [8], but they were ineffective in immune system cells pretreated with FAS-ligand [7]. Antiapoptotic effect of activators of cAMP signaling was noted in neutrophils [9], hepatocytes [10], and neuronal [11] and endothelial [12] cells but not in T-lymphocytes [13]; moreover, in thymocytes activation of cAMP signaling stimulated apoptosis [14].

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The antiapoptotic effect of Na^+/K^+-ATPase inhibitors on VSMC was abolished by adding inhibitors of RNA and protein syntheses [15]. Taking into consideration data on the sharp increase of [^{3}H]uridine incorporation into RNA fraction [15] and identification (by 2D-electrophoresis) of newly synthesized proteins [16], it was suggested that slowing VSMC death at the increased intracellular $[Na^+]/[K^+]$ ratio is mediated by activation of expression of genes encoding apoptosis inhibitor. In many cell types cAMP increase is accompanied by expression of genes containing a Ca^{2+}-cAMP-response element (CRE) in their promoter [17-19]. Transfection of neuronal cells with CRE-binding protein (CREB) protected them against okadaic acid-induced apoptosis [20]. This effect may be attributed to increased expression of the gene encoding the anti-apoptotic protein bcl-2; its promoter contains CRE [21]. Antiapoptotic protection of hepatocytes by activation of cAMP signaling requires the presence of inhibitors of RNA synthesis [10]. In the present study we investigated the effect of adenylate cyclase and forskolin on RNA synthesis in VSMC and also effects of inhibitors of RNA and protein syntheses on the development of apoptosis during activation of cAMP-dependent signaling.

MATERIALS AND METHODS

Cell culture. Vascular smooth muscle cells were isolated from aortas of male Sprague–Dawley rats as described previously [22]. The resulting cells were transfected with E1A adenovirus by Dr. M. R. Bennett (University of Cambridge, UK). In contrast to intact VSMC, which are rather resistant to apoptosis triggers, from 30 to 50% E1A-transfected cells (VSMC-E1A) undergo apoptosis after the incubation in growth factor free medium for 4-6 h [23]. Therefore, these cells are a convenient model for the pharmacological analysis of the role of signal systems in the triggering and inhibition of apoptosis.

Detection of apoptosis. The development of apoptosis in VSMC-E1A was monitored by the accumulation of chromatin fragments [24]. Briefly, cells grown in 24-well plates for 24 h in DMEM (Dulbecco modified Eagle's medium) containing 10% calf serum and 1 μCi/ml [^{3}H]thymidine were washed and incubated in the medium without radiolabeled thymidine for 48 h. Apoptosis was initiated by transfer of the cells into the serum-free medium containing additions listed in the tables. After the incubation medium was collected for radioactivity counting (A_1) cells were lysed in the cold with 10 mM Tris-HCl buffer, pH 8.0, containing 0.5% Triton X-100 for 15 min. The resulting lysate was centrifuged for 10 min at 10,000 rpm; the supernatant was used for radioactivity counting (A_2). The sediment and remaining cells (in wells) were treated with 10% SDS and 4 mM EDTA and were also used for radioactivity counting (A_3). Relative content of intracellular chromatin fragments was determined as $A_2 \cdot (A_1 + A_2 + A_3)^{-1} \cdot 100\%$. In previous studies we demonstrated that this method is very convenient for quantitative evaluation of apoptosis and the data obtained well corresponded to analysis of DNA fragments, phosphatidylserine accumulation on the external surface of plasma membrane, and also to morphological analysis of the content of apoptotic cells [6, 7, 25]. In the other series the development of apoptosis was evaluated by caspase-3 activity measured by the rate of cleavage of the fluorescence-labeled selective substrate of this enzyme: Asp-Glu-Val-Asp-AMC. Caspase-3 activity was measured in the absence and in the presence of the selective inhibitor of this enzyme Ac-Asp-Glu-Val-Asp-CHO (1 μM) [6].

Synthesis of RNA and protein was determined by incorporation of [^{3}H]uridine and [^{3}H]leucine, respectively [26, 27]. The effect of RNA and protein synthesis inhibitors on cell viability was evaluated by the proportion of Trypan blue stained cells. Cell suspension obtained after the treatment with 0.05% trypsin in Mg^{2+}- and Ca^{2+}-free medium was treated with 0.2% Trypan blue. The total number of cells and the number of trypan blue stained cells were determined using a hematocytometer.

Protein content was determined by the Lowry method [28].

Identification of phosphorylated proteins using 2D-electrophoresis. Cells (VSMC-E1A) grown in flasks (85 cm^2) were preincubated in phosphate-free DMEM containing 10% serum and $^{32}P_i$ (200 μCi/ml) for 3 h. After this pretreatment the incubation medium was substituted for standard DMEM containing 10% serum, and the cells were incubated in the presence or absence of forskolin for 3 or 24 h. Cells were then washed four times with cold 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl and lysed in 600 μl of medium containing 8 M urea, 20 mM tributyl phosphine, 50 mM thiourea, 40 mM Tris, and 4% CHAPS. The resulting samples were applied onto lanes with nonlinear pH gradient (pH = 3-10) Immobiline (Pharmacia, Sweden) and subjected to isoelectrofocusing in a Protean AEF system (BioRad, USA) followed by subsequent electrophoresis (for 16 h) in 8-16% polyacrylamide gel (10 mA) using the Protean II system (BioRad). Gels were fixed in the mixture 50% methanol/10% acetic acid and dried under vacuum. Phosphorylated proteins were developed by radioautography and analyzed using PD Quest software (Bio-Rad).

Chemicals. [^{3}H]Thymidine, [^{3}H]uridine, [^{3}H]leucine, and ^{32}P-orthophosphate were purchased from Amersham (Canada). Other chemicals were obtained from Sigma (USA), BIOMOL (USA), and Gibco (USA).

RESULTS

We previously found that incubation of VSMC-E1A in growth factor-free medium for 3-6 h is accompanied by rapid triggering of apoptosis [6, 7, 23, 25]. The results of the present study are consistent with the previous observations. Figure 1 shows that incubation of VSMC-E1A in growth factor (serum)-free medium for 6 h was accompanied by significant increase in chromatin fragments from 2 to 25-30% (Fig. 1, curve *2*). Forskolin addition completely abolished the effect of serum deprivation on the accumulation of chromatic fragments during incubation for 6-12 h; after the incubation for 24 h the content of chromatin fragments in forskolin-treated cells was two-times less than in control cells (Fig. 1, curves *3* and *2*, respectively). Previously we found that besides forskolin, the activation of cAMP signaling by β-adrenoreceptor agonists, cholera toxin (G_s protein activator) phosphodiesterase inhibitor and cAMP analog, 8-Br-cAMP, also inhibited apoptosis in VSMC [6].

Transient suppression of apoptosis in cells with an activated cAMP-dependent signaling system may be a consequence of expression of genes encoding apoptosis inhibitor. It is also possible that this effect is due to involvement of protein kinase A in the phosphorylation of pre-existing regulators of the apoptotic machinery leading to modification of their activity. To test these alternative hypotheses, we compared effects of adenylate cyclase activator (forskolin) on apoptosis in the presence and absence of inhibitors of RNA synthesis (actinomycin D (Act-D)) and protein synthesis (cycloheximide (CHX)), respectively. In accordance with previous observations in other cell types [18, 19], the incubation of VSMC-E1A with forskolin for 2 h caused an increase of RNA synthesis (by 50-60%) (Table 1). Pretreatment with Act-D for 30 min was accompanied by 100-fold reduction of RNA synthesis in VSMC-E1A and complete abolishment of the effect of forskolin. Pretreatment with CHX caused 6-7-fold reduction of [^{3}H]leucine incorporation into the protein fraction of VSMC-E1A. Neither Act-D nor CHX influenced the development of apoptosis measured by accumulation of chromatin fragments in cells subjected to incubation in growth factor-free medium (serum deprivation) for 6 h (Table 2). Similar results were obtained during assay of caspase-3 activity (Table 3). Neither compound influenced the number of Trypan blue stained cells (Table 1). This suggests lack of toxic effect of the short-term inhibition of protein and RNA syntheses.

To test the possible role of cAMP-dependent protein phosphorylation in modulation of apoptosis we investigated the content of phosphorylated proteins in VSMC-E1A treated with forskolin for 3 and 24 h. Forskolin completely abolished the development of apoptosis during incubation for 6 h; the antiapoptotic effect of forskolin was reduced during longer incubation (for 12 h) (Fig. 1). Results of 2D-electrophoresis of ^{32}P-labeled cells indicate that incubation with forskolin for 3 h was accompanied by phosphorylation of more than 70 proteins of molecular masses ranging from 15 to 200 kD and p*I* from 3.2 to 9.5 (Fig. 2b); after the incubation with forskolin for 24 h, the number of phosphorylated proteins was reduced to 20-30 (Fig. 2c).

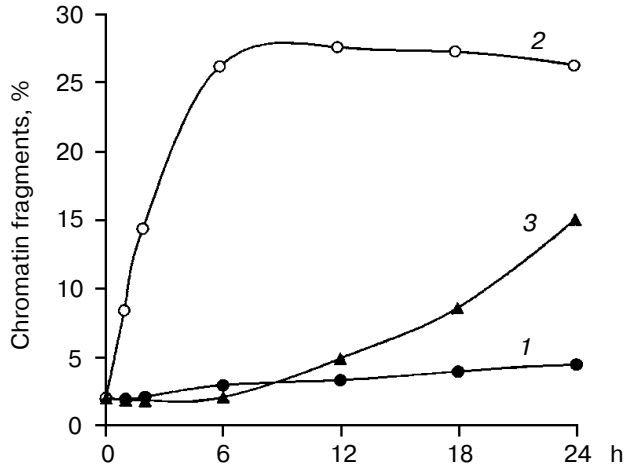

Fig. 1. Kinetics of accumulation of intracellular chromatin fragments in VSMC transfected with E1A-adenovirus and incubated in growth factor-free medium: *1*) control (incubated with 10% calf serum); *2*) serum-free medium; *3*) serum-free medium + 10 μM forskolin. Total content of ^{3}H-labeled DNA was defined as 100%. The data are the means of four measurements.

DISCUSSION

Previous studies revealed that the activation of the cAMP signaling system and the increase of intracellular ratio of Na^+/K^+ by Na^+/K^+-ATPase inhibitors caused transient inhibition of apoptosis in VSMC at the stage preceding caspase-3 activation [6, 7]. Inhibitors of RNA and protein synthesis abolished the effect of Na^+/K^+-ATPase inhibitors on the development of apoptosis [15]. The latter suggests that inhibition of apoptosis by inversion of $[Na^+]_i/[K^+]_i$ is realized by the activation of some $[Na^+]/[K^+]$-sensor (S) followed by subsequent expression of an unidentified inhibitor of apoptosis (INH) (Fig. 3). It was also noted that the inhibition of Na^+/K^+-ATPase in VSMC caused 5-10-fold increase of RNA synthesis [15]. The latter may also be related to expression of early response genes (ERG). In fact, it was found that inhibition of Na^+/K^+-ATPase in hepatocytes [29], lymphocytes [30], fibroblasts [31] and cardiomyocytes [32] was accompanied by sharp increase of c-fos and c-jun mRNA. The role of these and other ERG as possible triggers of expression of apoptosis inhibitor in VSMC (Fig. 3) are currently being investigated in our laboratory.

Table 1. Effect of actinomycin D and cycloheximide on synthesis of RNA and protein and viability of VSMC transfected with E1A adenovirus

Additions to incubation medium, μg/ml	RNA synthesis, cpm/mg protein		Protein synthesis, cpm/mg protein	Trypan blue stained cells, %
	control	forskolin		
Control	43876 ± 3412	69344 ± 5733	8754 ± 988	5.6 ± 0.7
Actinomycin D, 2	471 ± 68*	418 ± 177*	2538 ± 311*	6.4 ± 0.5
Cycloheximide, 2	9213 ± 700*	10287 ± 1075*	1313 ± 98*	6.6 ± 1.1

Note: VSMC-E1A cells were cultivated in the presence of 10% calf serum. Rates of RNA and protein syntheses were evaluated by incorporation of [^{3}H]uridine and [^{3}H]leucine (1 μCi/ml); the radiolabeled compounds were added 2 h before termination of cell incubation. Actinomycin D and cycloheximide were added 30 min before addition of forskolin (10 μM) and isotopes. Trypan blue stained cells are given as percent of total number of cells. Data represent the mean ± SD of four measurements.

* $p < 0.001$ compared to control.

Table 2. Effect of forskolin, actinomycin D, and cycloheximide on apoptosis in VSMC-E1A measured by accumulation of intracellular chromatin fragments

Additions to incubation medium, μg/ml	Content of chromatin fragments, %			
	medium with 10% serum		serum-free medium	
	control	forskolin	control	forskolin
Control	2.4 ± 0.3	1.9 ± 0.2	28.7 ± 3.6	3.3 ± 1.0**
Actinomycin D, 2	3.3 ± 0.6	2.1 ± 0.3	25.4 ± 2.0	4.0 ± 0.5**
Cycloheximide, 2	3.6 ± 0.4	2.0 ± 0.3*	26.0 ± 2.7	3.8 ± 0.4**

Note: VSMC-E1A cells pre-labeled with [^{3}H]thymidine were cultivated in medium containing 10% calf serum, then cells were incubated for 6 h in the presence or absence of serum and 10 μM forskolin. Actinomycin D and cycloheximide were added 30 min before forskolin addition. Total content of ^{3}H-labeled DNA was defined as 100%. Data represent mean ± SD of four measurements.

* $p < 0.02$;

** $p < 0.001$ compared to cells incubated without forskolin.

Table 3. Effect of forskolin, actinomycin D, and cycloheximide on the activity of caspase-3 measured in VSMC-E1A cells

Additions to incubation medium, μg/ml	Caspase-3 activity, nmole/h per mg protein			
	medium with 10% serum		serum-free medium	
	control	forskolin	control	forskolin
Control	0.42 ± 0.16	0.31 ± 0.10	4.7 ± 1.2	0.55 ± 0.11*
Actinomycin D, 2	0.38 ± 0.12	0.33 ± 0.11	4.1 ± 0.9	0.48 ± 0.17*
Cycloheximide, 2	0.40 ± 0.13	0.35 ± 0.09	5.0 ± 1.1	0.44 ± 0.14*

Note: VSMC-E1A cells were cultivated in medium containing 10% calf serum, then cells were incubated for 6 h in the presence or absence of serum and 10 μM forskolin. Actinomycin D and cycloheximide were added 30 min before forskolin addition. Data represent mean ± SD of four measurements.

* $p < 0.001$ compared to cells incubated without forskolin.

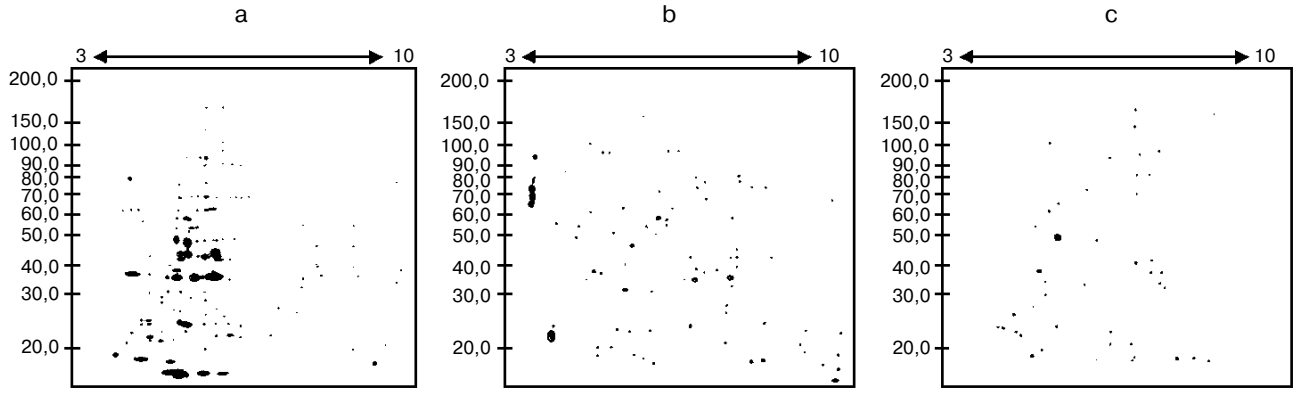

Fig. 2. Effect of forskolin on protein phosphorylation in VSMC transfected with E1A adenovirus: a) proteins phosphorylated under control conditions (10% calf serum, no forskolin); b) proteins phosphorylated after incubation with forskolin (10 μM) for 3 h; c) proteins phosphorylated after incubation with forskolin (10 μM) for 24 h; p*I* and M_r of phosphoproteins are given on abscissa and ordinate, respectively.

Results of the present study clearly indicate that in contrast to Na^+/K^+-ATPase inhibitors the inhibition of apoptosis during activation of cAMP-dependent signaling is accompanied by moderate increase of [^{3}H]uridine incorporation into RNA fraction (Table 1) and does not depend on the presence of inhibitors of RNA and protein synthesis (Tables 2 and 3). This suggests that the anti-apoptotic effect of cAMP is not related to expression of genes encoding an inhibitor of programmed cell death. Results of the present study also suggest that cAMP-dependent inhibition of apoptosis involves activation of protein kinase A followed by phosphorylation of pre-existing proteins. This may have two possible consequences: inhibition of activity of apoptosis triggers (ACT~P) or activation of inhibitors of apoptotic machinery ((INH~P)*, Fig. 3). This is consistent with the fol-

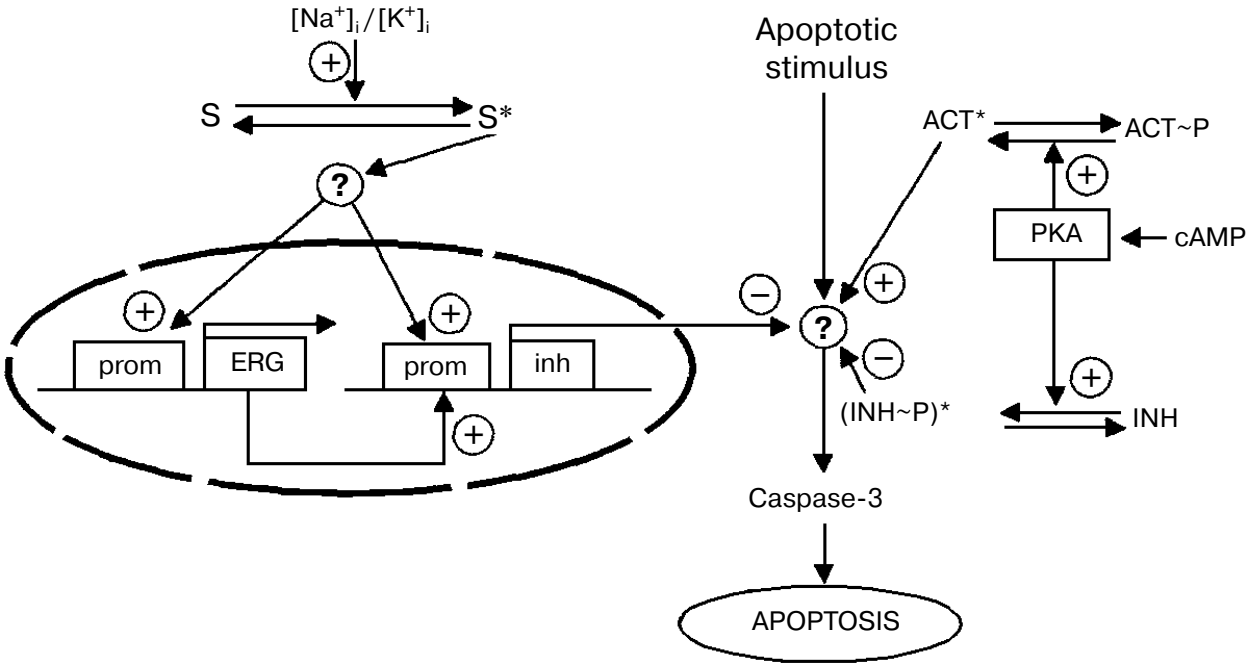

Fig. 3. Possible mechanisms of involvement of $[Na^+]_i/[K^+]_i$ and cAMP in the inhibition of apoptosis in VSMC. S, sensor generating a signal during increase in intracellular $[Na^+]/[K^+]$; prom, promoter; ERG, early response genes; inh, gene encoding apoptosis inhibitor; PKA, protein kinase A; ACT~P and INH~P, phosphorylated forms of activator and inhibitor of apoptosis; *, activated state of proteins; + and –, stimuli leading to activation and inhibition of the signal cascade, respectively; ?, unidentified component of the signal cascade. Other details are given in the text.

lowing data: 1) transfection of catalytic subunit of protein kinase A inhibited apoptosis of VSMC-E1A [6]; 2) addition of protein kinase A inhibitors attenuated antiapoptotic effect of activators of cAMP-signaling system [6]; 3) transient apoptosis inhibition by forskolin (Fig. 1) correlated with kinetics of protein kinase A activation [6] and protein phosphorylation (Fig. 2).

As in the case of other cell types, inhibitors of phosphatidylinositol-3 (PI3) kinase attenuated the protective effect of growth factors against the development of apoptosis in VSMC [5, 33]. Serine/threonine protein kinase B (ACT) is one of substrates of PI3 kinase. Recently it was found that phosphorylation of a serine and threonine residue of bcl-2 significantly increased its antiapoptotic function [34, 35], whereas Bad phosphorylation reduced efficacy of this protein as a trigger of apoptosis [36]. Bad [36] and procaspase-9 [37] are phosphorylated by ACT *in vivo*. In various cell types protein kinase A activates ACT via a PI3 kinase-dependent [10] and -independent mechanisms [38]. However, it should be noted that in hepatocytes protection against apoptosis by activation of cAMP-signaling system does not involve ACT activation [10]. The role of this cascade leading to phosphorylation of Bad and procaspase-9 and also the role of phosphoproteins visualized by 2D-electrophoresis (Fig. 2) in the inhibition of apoptosis during activation of cAMP-dependent signaling in VSMC requires further investigation.

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REFERENCES

1. Hamet, P. (1995) *Curr. Opin. Nephrol. Hypert.*, **4**, 1-7.
2. DeBlois, D., Orlov, S. N., and Hamet, P. (1999) in *Apoptosis in Cardiac Biology* (Schunkert, H., ed.) Kluwer Academic Publishers, Boston-Doredrecht-London, pp. 213-222.
3. Bennett, M. R., Littlewood, T. D., Schwartz, S. M., and Weissberg, P. L. (1997) *Circ. Res.*, **81**, 591-598.
4. Geng, Y.-J. (1997) *Heart and Vessels*, **12**, 76-80.
5. Orlov, S. N., DeBlois, D., Tremblay, J., and Hamet, P. (1999) *Cardiovasc. Risk Factors*, **9**, 67-79.
6. Orlov, S. N., Thorin-Trescases, N., Dulin, N. O., Dam, T.-V., Fortuno, M. A., Tremblay, J., and Hamet, P. (1999) *Cell Death Differ.*, **6**, 672-691.
7. Orlov, S. N., Thorin-Trescases, N., Kotelevtsev, S. V., Tremblay, J., and Hamet, P. (1999) *J. Biol. Chem.*, **274**, 16545-16552.
8. Isaev, N. K., Stelmashook, E. V., Halle, A., Harms, C., Lautenschlager, M., Weih, M., Dirnagl, U., Victorov, I. V., and Zorov, D. B. (2000) *Neurosci. Lett.*, **283**, 41-44.
9. Parvathenani, L. K., Buescher, E. S., Chacon-Cruz, E., and Beebe, S. J. (1998) *J. Biol. Chem.*, **273**, 6736-6743.
10. Li, J., Yang, S., and Billiar, T. R. (2000) *J. Biol. Chem.*, **275**, 13026-13034.
11. Galli, G., Meucci, O., Scorziello, A., Werge, T. M., Calissano, P., and Schettini, G. (1995) *J. Neurosci.*, **15**, 1172-1179.
12. Polte, T., and Schroder, H. (1998) *Biochem. Biophys. Res. Commun.*, **251**, 460-465.
13. Sciorati, C., Rovere, P., Ferrarini, M., Helta, S., Manfredi, A. A., and Clementi, E. (1997) *J. Biol. Chem.*, **272**, 23211-23215.
14. Suzuki, K., Takaduma, T., and Kizaki, H. (1991) *Cell. Immunol.*, **134**, 235-240.
15. Orlov, S. N., Taurin, S., Thorin-Trescases, N., Dulin, N. O., Tremblay, J., and Hamet, P. (2000) *Hypertension*, **35**, 1062-1068.
16. Taurin, S., Orlov, S. N., Seyrantepe, V., Hamet, P., and Pshezhetsky, A. V. (2000) *Can. J. Cardiol.*, **16**, 153F.
17. Tamai, K. T., Monaco, L., Nantel, F., Zazopoulos, E., and Sassone-Corsi, P. (1997) *Recent Prog. Hormone Res.*, **52**, 121-139.
18. Yao, H., York, R. D., Misra-Press, A., Carr, D. W., and Stork, P. J. S. (1998) *J. Biol. Chem.*, **273**, 8240-8247.
19. Grieco, D., Porecllini, A., Avvedimento, E. V., and Gottesman, M. E. (1996) *Science*, **271**, 1718-1723.
20. Wilson, B. E., Mochon, E., and Boxer, L. M. (1996) *Mol. Cell Biol.*, **16**, 5546-5556.
21. Walton, M., Woodgate, A. M., Muravlev, A., Xu, R., During, M. J., and Dragunow, M. (1999) *J. Neurochem.*, **73**, 1836-1842.
22. Orlov, S. N., Tremblay, J., and Hamet, P. (1996) *Am. J. Physiol.*, **270**, C1388-C1397.
23. Bennett, M. R., Evan, G. I., and Schwartz, S. M. (1995) *Circ. Res.*, **77**, 266-273.
24. Orlov, S. N., Dam, T. V., Tremblay, J., and Hamet, P. (1996) *Biochem. Biophys. Res. Commun.*, **221**, 708-715.
25. Champagne, M.-J., Dumas, P., Orlov, S. N., Bennett, M. R., Hamet, P., and Tremblay, J. (1999) *Hypertension*, **33**, 906-913.
26. Andrawis, N. S., and Abernethy, D. R. (1993) *Am. J. Med.*, **306**, 137-140.
27. Giasson, E., Servant, M. J., and Meloche, S. (1997) *J. Biol. Chem.*, **272**, 26879-26886.
28. Hartee, E. I. (1972) *Analyt. Biochem.*, **48**, 422-427.
29. Cayanis, E., Russo, J. J., Wu, Y., and Edelman, I. S. (1992) *J. Membr. Biol.*, **125**, 163-170.
30. Olej, B., dos Santos, N. F., Leal, L., and Rumjanek, V. M. (1998) *Biosci. Rep.*, **18**, 1-7.
31. Nakagawa, Y., Rivera, V., and Larner, A. C. (1992) *J. Biol. Chem.*, **267**, 8785-8788.
32. Peng, M., Huang, L., Xie, Z., Huang, W.-H., and Askari, A. (1996) *J. Biol. Chem.*, **271**, 10372-10378.
33. Bai, H., Pollma, M. J., Inishi, Y., and Gibbons, G. H. (1999) *Circ. Res.*, **85**, 229-237.
34. Horiuchi, M., Hayashida, W., Kambe, T., Yamada, T., and Dzau, V. J. (1997) *J. Biol. Chem.*, **272**, 19022-19026.
35. Ito, T., Deng, X., Carr, B., and May, W. S. (1997) *J. Biol. Chem.*, **272**, 11671-11673.
36. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell*, **91**, 231-241.
37. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science*, **282**, 1318-1321.
38. Filippa, N., Sable, C. L., Filloux, C., Hemmings, B., and van Obberghen, E. (1999) *Mol. Cell Biol.*, **19**, 4989-5000.