Stimulation of intracellular topoisomerase I activity by vasopressin and thrombin

Differential regulation by pertussis toxin

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Incubation of cultured rat aortic smooth muscle cells (A-10, ATCC CRL 1476) with [8-arginine]vasopressin (AVP) or thrombin increased the amount of DNA strand breakage induced by camptothecin, an inhibitor of topoisomerase I (DNA topoisomerase; EC 5.99.1.2) and transiently stimulated the extractable activity of this enzyme. Both topoisomerase-related responses were prevented by treatment of the cells with AVP or thrombin plus the appropriate receptor antagonist. The increase in strand breakage mediated by AVP and thrombin depended on the concentration of hormone. Neither AVP nor thrombin had any effect on strand breaks obtained with the epipodophyllotoxin VM-26, an inhibitor of topoisomerase II [DNA topoisomerase (ATP-hydrolysing); EC 5.99.1.3]. Pretreatment of the cells with pertussis toxin partially inhibited thrombin-mediated increases in camptothecin-induced strand breakage whereas AVP-mediated increases were unaffected. These results are consistent with the notion that AVP and thrombin induce a transient increase in intracellular topoisomerase I activity via interactions with their respective cell surface receptors and that the effects of the activation of these receptors are mediated by different G-proteins.

INTRODUCTION

The neurohypophyseal hormone vasopressin mediates its physiological responses by interacting with at least two types of receptors, the vascular V_1 and renal V_2 receptors [1]. V_2 receptors are positively coupled to adenylate cyclase and mediate antidiuretic activity in the kidney [2–4]. V_1 receptor signal transduction is effected by activation of phosphoinositide-specific phospholipase C, which liberates diacylglycerol (DAG) and inositol phosphates and increases intracellular calcium release [5–7]. V_1 receptor activation also results in direct, rapid release of arachidonic acid [8]. Our laboratory has been interested in the signal transduction pathways activated by vasopressin receptors. For this purpose we have used a model system A-10 (ATCC CRL 1476), a smooth muscle cell line derived from rat thoracic aorta that displays a high density of vasopressin V_1 receptors [9]. These vasopressin receptors mediate vascular contraction by activating phosphoinositide-specific phospholipase C, and generating inositol 1,4,5-trisphosphate and DAG [5]. We have suggested that [8-arginine]vasopressin (AVP) might also enhance vasoconstriction by inhibiting the vasorelaxation mediated by β -adrenergic agonist and atrial natriuretic factor since the addition of AVP to these cells results in the inhibition of β -adrenergic receptor agonist and atrial natriuretic factor-induced cyclic AMP and cyclic GMP accumulation respectively [10,11]. It has been shown in quite a few systems that phosphatidylinositol (PI) turnover elevates cytosolic calcium and DAG levels which in turn can synergistically activate protein kinase C. These events result in a rapid expression of certain oncogenes including *c-myc* and

c-fos [12–14]. We have also shown that exposure of A-10 cells to AVP results in the rapid induction of *c-fos* oncoprotein without affecting the levels of *c-myc* oncoprotein [14a]. Since these oncogenes code for DNA-binding proteins, it was of interest to determine whether any nuclear enzymes, for example topoisomerases, are involved in AVP-mediated effects on gene transcription.

Topoisomerase I (DNA topoisomerase; EC 5.99.1.2) and II [DNA topoisomerase (ATP-hydrolysing), EC 5.99.1.3] are classes of enzymes which catalyse interconversions via transient single or double strand breaks in DNA [15,16] resulting in the regulation of DNA metabolism, including transcription [17–20]. Since topoisomerase II has been shown to be phosphorylated [21] by protein kinase C *in vitro* [22], we have hypothesized that receptor agonists such as AVP might modulate these enzymes via the calcium–DAG pathway.

We now report that interactions of AVP with V_1 receptors resulted in transient stimulation of topoisomerase I activity in A-10 cells. In addition, topoisomerase I was stimulated by incubating the cells with thrombin, which also stimulates PI turnover in these cells (P. Nambi, H. L. Wu, N. Aiyar & S. T. Crooke, unpublished work). We also present data to demonstrate that the responses mediated by AVP and thrombin were differentially regulated by pertussis toxin pretreatment of the cells. These data are in agreement with PI-turnover data which suggest that thrombin-mediated PI turnover involves a pertussis toxin-sensitive guanine-nucleotide-binding regulatory protein (G-protein), whereas AVP-mediated PI turnover involves a pertussis toxin-insensitive G-protein.

Abbreviations used: DAG, diacylglycerol; AVP, [8-arginine]vasopressin; PI, phosphatidylinositol; DdAVP, [1-deamino, 8-arginine]vasopressin.

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MATERIALS AND METHODS

Chemicals and radioisotopes

Camptothecin (NSC 94600) was obtained from the National Cancer Institute, Bethesda, MD, U.S.A. AVP was purchased from Bachem, Torrance, CA, U.S.A.; thrombin from Sigma, St. Louis, MO, U.S.A.; pertussis toxin from List Biologicals, Campbell, CA, U.S.A.; [1-deamino,8-D-arginine]vasopressin (DdAVP) and SK&F 100273 were kindly provided by the peptide chemists at SK&F Laboratories. [*methyl*-³H]Thymidine (78 Ci/mmol) and [*methyl*-¹⁴C]thymidine (52 mCi/mmol) were purchased from New England Nuclear Corp, Boston, MA, U.S.A.

Cell culture

A-10 cells (ATCC CRL 1476) were obtained from the American Type Culture Collection. They were maintained routinely in culture as monolayers at 37 °C in Dulbecco's modified Eagle's medium containing 20% fetal calf serum. Before the initiation of the experiments, the cells were subcloned twice by limiting dilution. All experiments were performed with cells passaged once a week for no more than 4 months. For elution experiments, culture wells (35 mm diam. 6-well plates) were seeded with 1 ml of medium containing 7.5×10^4 cells. For nucleus extraction experiments, 150 cm² plastic tissue culture flasks were seeded with 20 ml of medium containing 1.5×10^6 cells. Experiments were performed after 3 days.

Determination of DNA strand breakage by alkaline elution

DNA single strand breaks produced by camptothecin (topoisomerase I-linked) were assayed by the alkaline elution method of Kohn *et al.* [23], described in detail in a previous publication [24]. Cells (2×10^5 /well) were labelled uniformly in their DNA with [*methyl*-¹⁴C]-thymidine (0.02 μ Ci/ml) for approx. one cell doubling before being treated with AVP or thrombin for 10 min at 37 °C. Camptothecin was added from stock (dissolved in dimethyl sulphoxide) to final concentrations of 0.2–5 μ M and the cells were incubated further for 60 min at 37 °C. The reaction was stopped by aspirating the incubation medium and the cells were scraped into 5 ml of Dulbecco's phosphate-buffered saline at 4 °C and loaded on to polycarbonate membrane filters, lysed (in the presence of 0.5 mg of proteinase K/ml) and eluted as described previously [24]. Fractions were collected every 3 h. Strand breakage was expressed quantitatively as 'rad equivalents' [23].

Preparation of nucleus extracts; assay for topoisomerase I activity

Cells in T-150 flasks were washed twice with Dulbecco's phosphate-buffered saline supplemented with 0.2% bovine serum albumin, 10 mM-MgCl₂ and 0.1% glucose (DPBS²⁺) and incubated at 37 °C in the same buffer with or without the test compounds for the indicated times. At the end of incubation the cells were washed twice with cold DPBS²⁺, scraped and centrifuged at 1500 *g* for 10 min at 4 °C. The pelleted cells were swollen in 1 ml of hypo-osmotic buffer (5 mM-KH₂PO₄, pH 7.0/2 mM-MgCl₂/1 mM-phenylmethanesulphonyl fluoride/1 mM-2-mercaptoethanol/0.1 mM-EDTA) for 30 min at 4 °C and then disrupted by 30 strokes with a

Dounce homogenizer. Nuclei were collected by centrifugation (200 *g*, 10 min) and resuspended at 3×10^7 cells/ml in iso-osmotic buffer (5 mM-KH₂PO₄, pH 7.5/100 mM-NaCl/10 mM-2-mercaptoethanol/0.5 mM-phenylmethanesulphonyl fluoride). NaCl (5 M) was added to a final concentration of 0.35 M and the nuclei were vortexed gently, incubated on ice for 30 min and sedimented (1000 *g* for 10 min). The supernatant was assayed for topoisomerase I activity by monitoring the relaxation of supercoiled pBR322 DNA in the absence of ATP. Portions (5 μ l) of diluted extracts were mixed with 5 μ l of supercoiled pBR322 DNA. The reactions were allowed to proceed for 30 min at 37 °C, after which the DNA was electrophoresed in 1.0% agarose gels in TBE buffer. DNA was revealed by staining with Ethidium Bromide after electrophoresis. One unit of topoisomerase I activity was defined as the amount that produced observable relaxation of 100 ng of the supercoiled DNA substrate. Specific activities were estimated by dividing the activity (unit/ml) by the protein concentration of the extracts, determined by the method of Bradford [25].

RESULTS AND DISCUSSION

Topoisomerase activity *in vivo* was assayed by two techniques, each of which gives an estimate of intracellular enzyme activity. It has been shown that camptothecin-induced cellular DNA strand breakage is an indicator of intracellular topoisomerase I [20] and VM-26-induced DNA strand breakage is an indicator of intracellular topoisomerase II activity [18]. Topoisomerase

Fig. 1. Increase in topoisomerase I-associated DNA single strand breakage by AVP

A-10 cells in 6-well plates were prelabelled with [¹⁴C]thymidine and treated without (○) and with (●) 1 μ M-AVP for 10 min at 37 °C. To these were added increasing concentrations of camptothecin. Incubation was continued for 60 min at 37 °C. The strand breakage was measured as explained in the Materials and methods section. The data presented are means $\pm$ S.E.M. from three independent determinations. Points without error bars represent values whose S.E.M. are contained within the diameter of the symbols.

Fig. 2. Dose dependence of the increases in topoisomerase I-associated DNA strand breakage mediated by AVP (a) or thrombin (b)

Increasing concentrations of AVP (a) or thrombin (b) were added to [^{14}C]thymidine-labelled A-10 cells and DNA single strand breakage induced by 5 μM -camptothecin was determined as explained in the Materials and methods section. The data presented are means of duplicate determinations from one experiment which is representative of two experiments.

merase I [19] and II [18] protein-linked breaks have been demonstrated to be associated with induced gene activity. Incubation of A-10 cells with 1 μM -AVP for 10 min before the addition of camptothecin increased the yield of camptothecin-induced lesions as shown in Fig. 1. In similar experiments we were unable to demonstrate, using VM-26, any increase in topoisomerase II-associated DNA strand breakage with AVP (results not shown).

Vasopressin increased camptothecin-induced lesions in a concentration-dependent manner, as shown in Fig. 2(a). The concentration of AVP required to cause 50% of maximal response was 6 nM, which compares very well with the K_d of vasopressin either for binding to these cell surface receptors [9] or for inducing the accumulation of inositol 1,4,5-trisphosphate and DAG [5] or mobilization of calcium. Thrombin is another hormone that induces accumulation of inositol trisphosphate and DAG in these cells (P. Nambi, H. L. Wu, N. Aiyar & S. T. Crooke, unpublished work). Fig. 2(b) shows the concentration-response effect of thrombin on camptothecin-induced DNA strand breakage. The half-maximal concentration of thrombin for increasing the number of camptothecin-induced DNA breaks was approx. 8 ng/ml. This compares well with the half-maximal concentration of

Table 1. Camptothecin-induced DNA single strand breakage: effects of AVP, SK&F 100273 (V_1 antagonist) and DdAVP

The data presented are averages of two independent observations from a single experiment which is representative of three experiments.

Condition	DNA single strand breaks (rad equivalents)
Camptothecin alone (5 μM)	315
+ AVP (1 μM)	565
+ SK&F 100273 (1 μM)	315
+ AVP (1 μM) + SK&F 100273 (1 μM)	256
+ DdAVP (1 μM)	315

thrombin (30–50 ng/ml) required to stimulate inositol phosphate accumulation and calcium release in these cells (P. Nambi, H. L. Wu, N. Aiyar & S. T. Crooke, unpublished work).

The vasopressin-mediated increase in camptothecin-induced DNA strand breakage was inhibited by SK&F 100273, a V_1 selective antagonist [26] (Table 1), suggesting that this effect of AVP was mediated by V_1 receptors. This is further supported by the observation that DdAVP, a V_2 selective agonist [27], was ineffective in increasing camptothecin-induced strand breakage (Table 1).

Extractable topoisomerase I activity also was increased by thrombin treatment. The maximum increase was observed 5–30 min after the addition of agonist (Fig. 3a). Thereafter, the activity decreased as a function of time of incubation with thrombin. After 60 min, topoisomerase I activity extracted from thrombin-treated cells was indistinguishable from that from untreated cells. Hirudin, a thrombin antagonist, prevented the thrombin-induced increase in topoisomerase I activity (Fig. 3a). Similar results were also obtained when AVP was used instead of thrombin, as shown in Fig. 3(b). SKF 100273, a V_1 antagonist, prevented the AVP-induced increase in topoisomerase I activity. (Fig. 3b). Neither AVP nor thrombin had any effect on extractable topoisomerase II activity (results not shown). Thus, by two independent assays we have demonstrated that PI-turnover-coupled cell surface receptors activate a nuclear enzyme, topoisomerase I. We believe that topoisomerase I is activated either by protein kinase C-catalysed phosphorylation or by a third protein which was activated by protein kinase C. Our data do not permit us to distinguish between these two possibilities. We note that topoisomerase II activity was not altered by AVP or thrombin binding in these cells. Thus, the regulation of topoisomerase activity by cellular signalling mechanisms may depend upon cell type.

We have previously demonstrated that vasopressin receptors in A-10 cells are coupled to G-proteins [9,28] and that the AVP-induced PI turnover and calcium mobilization are not affected by pertussis toxin pretreatment of the cells. To further validate that the effects of AVP on topoisomerase I are mediated by receptor binding coupled to PI turnover and to compare them with the activities of thrombin, we tested the sensitivity of the observed AVP-mediated increase in intracellular topoisomerase I activity to pertussis toxin

Fig. 3. Increase in extractable topoisomerase I activity caused by treatment of A-10 cells with thrombin (a) or AVP (b)

A-10 cells were treated with 5 μg of thrombin/ml (a) or 1 μM-AVP (b) in the presence (●) or absence (■) of receptor antagonists (5 μg of hirudin/ml for thrombin; 10 μM-SK&F 100273 for AVP). After the indicated times, topoisomerase I activity contained in nuclear extracts of the cells was determined as described in the Materials and methods section. Data are from one experiment representative of three.

pretreatment. ADP-ribosylation of these cell membranes with [³²P]NAD and pertussis toxin results in the specific incorporation of [³²P]ADP-ribose into a peptide of approx. *M_r* 39 000 [29]. Pretreatment of the cells with 1 μg of pertussis toxin/ml results in 94–96 % inhibition of [³²P]ADP-ribosylation of the membranes [29]. As shown in Table 2, pertussis toxin pretreatment of A-10 cells had no effect on the AVP-mediated response but partially inhibited the thrombin-mediated response. These data are in good agreement with our previous observation that, while AVP-mediated PI turnover was insensitive, thrombin-mediated PI turnover was inhibited between 50–70 % by pertussis toxin pretreatment (P. Nambi, H. L. Wu, N. Aiyar & S. T. Crooke, unpublished work). Taken together, our results strongly suggest that AVP and thrombin activate topoisomerase I via PI turnover, which is linked to two different G-proteins for the two agonists. It is not clear at this time why the inhibition by pertussis toxin is only partial even though the same

Table 2. Effect of pertussis toxin (PT) pretreatment on AVP- (1 μM) and thrombin- (5 μg/ml) mediated increases in topoisomerase I-linked DNA strand breakage

The data presented are averages of two independent observations in two separate experiments. Camptothecin concentration was 3 μM.

Condition	rad equivalents		Increase (%)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Control cells:				
Camptothecin alone	452	239		
+ AVP	626	340	39	42
+ Thrombin	946	463	109	94
PT-treated cells:				
Camptothecin alone	452	239		
+ AVP	622	365	38	53
+ Thrombin	724	356	60	49

concentration of toxin inhibits [³²P]ADP-ribosylation by as much as 95 % [29]. A possible explanation is that thrombin may mediate its response by two different G-proteins, only one of which is sensitive to pertussis toxin. The modulation of intracellular topoisomerase I activity by protein kinase C which is itself activated by cellular signalling mechanisms may be a means of coarse control over transcription. This mechanism may be common to agonists involved in PI turnover and other inducers of transcription that activate protein kinase C. To our knowledge this is the first demonstration of a coupling of cell surface receptors to a nuclear enzyme involved in the regulation of transcription and translation.

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