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27. Control of Aldosterone Secretion

ANGIOTENSIN II RECEPTORS AND MECHANISMS OF ACTION IN ADRENAL GLOMERULOSA CELLS

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Summary—The plasma-membrane receptors, coupling mechanisms, and effector enzymes that mediate target-cell activation by angiotensin II (AII) have been characterized in rat and bovine adrenal glomerulosa cells. The AII holoreceptor is a glycoprotein of Mr ~ 125,000 under non-denaturing conditions. Photoaffinity labeling of AII receptors with azido-AII derivatives has shown size heterogeneity among the AII binding sites between species and target tissues, with M_r values of 55,000 to 79,000. Such variations in molecular size probably reflect differences in carbohydrate content of the individual receptor sites. The adrenal AII receptor, like that in other tissues, is coupled to the inhibitory guanine nucleotide inhibitory protein (N_i). However, studies with pertussis toxin have shown that stimulation of aldosterone production by AII is not mediated by N_i but by a pertussis-insensitive nucleotide regulatory protein of unidentified nature. Although N_i is not involved in the stimulatory action of AII on steroidogenesis, it does mediate the inhibitory effects of high concentrations of AII upon aldosterone production. The actions of AII on adrenal cortical function are thus regulated by at least two guanine nucleotide regulatory proteins that are selectively activated by increasing AII concentrations.

The principal effector enzyme in AII action is phospholipase C, which is rapidly stimulated in rat and bovine glomerulosa after AII receptor activation. AII-induced breakdown of phosphatidylinositol bisphosphate (PIP_2) and phosphatidylinositol phosphate (PIP) leads to formation of inositol 1,4,5-trisphosphate (IP_3) and inositol 1,4-bisphosphate (IP_2). These are metabolized predominantly to inositol-4-monophosphate, which serves as a marker of polyphosphoinositide breakdown, whereas inositol-1-phosphate is largely derived from phosphatidylinositol hydrolysis. The AII-stimulated glomerulosa cell also produces inositol 1,3,4-trisphosphate, a biologically inactive IP_3 isomer formed from Ins-1,4,5-trisphosphate via inositol tetrakisphosphate (IP_4) during ligand activation in several calcium-dependent target cells. The Ins-1,4,5-P_3 formed during AII action binds with high affinity to specific intracellular reeceptors that have been characterized in the bovine adrenal gland and other AII target tissues, and may represent the sites through which IP_3 causes calcium mobilization during the initiation of cellular responses.

INTRODUCTION

Angiotensin II (AII) exerts an extraordinary diversity of actions in its multiple target tissues, which include the adrenal, liver, smooth muscle, heart, brain and kidney. Many of the actions of AII are concerned with cardiovascular regulation and fluid homeostasis, which are controlled by the integrated effects of AII in the nervous system and peripheral effector organs. In all of these locations, the cellular actions of AII are mediated by specific plasma-membrane receptors, which are usually coupled to phosphoinositide hydrolysis and initiate elevation of the cytoplasmic calcium concentration. The AII receptors of the adrenal glomerulosa cell were among the first to be identified, and their structural properties and activation mechanisms are still being clarified. This report describes recent developments in the physicochemical characterization of the adrenal AII receptor, and studies on its coupling to guanyl nucleotide regulatory proteins and phospholipase C-catalyzed hydrolysis of inositol phospholipids that serve as the source of intracellular signals responsible for target-cell activation.

EXPERIMENTAL

Properties of photoaffinity-labeled AII receptors

Plasma-membrane receptors for angiotensin II (AII) have been characterized by radioligand binding analysis and pharmacological studies in numerous target tissues of the octapeptide. The AII receptors in various tissues have similar binding affinities and comparable specificities for AII and its analogs, but individual target cells may exhibit diverse biological responses to AII agonist and antagonist derivatives [1]. The elucidation of mechanisms coupling AII binding to cellular activation requires more detailed analysis of the structural properties of the AII receptor in specific target cells, including adrenal, smooth muscle, liver, and nervous system. The physical characterization of AII receptor sites has been complicated by the difficulty of solubilizing the receptor in a sufficiently stable and active form to permit its analysis by affinity chromatography and other fractionation methods. For this reason, both photoaffinity labeling and radioligand cross-linking have been employed prior to AII receptor solubilization and characterization [2–5].

In studies using the azidobenzoyl derivative of [^{125}I]AII as the photoreactive radioligand to characterize adrenal and uterine AII receptors [2], SDS–PAGE analysis revealed two binding species of M_r 126,000 and 65,000 or 68,000. These findings suggested that the AII holoreceptor is a dimer composed of two ~65 kDa subunits. The similar M_r values of dog adrenal (65,000) and uterine (68,000) binding sites also suggested that the AII receptor is of comparable size in different target tissues. This observation was supported by subsequent reports on the apparent molecular weights of AII receptors from bovine adrenal (60,000 [3]) and rabbit liver (68,000 [6]). However, more recent studies with the carboxyterminal-azido analog, [Sar^1, (4-N_3)-Phe^8]AII [3], which binds with high affinity to AII receptors in adrenal membranes, have revealed several differences in the properties of AII receptors from individual species and target tissues [7]. An example of covalent labeling after photolysis of the receptor-bound ligand, with irreversible binding of over 50% of the analog bound to bovine adrenal receptors, is shown in Fig. 1. The C-terminal azido AII derivative has higher efficiency for labeling AII receptors than *N*-terminal azidobenzoyl AII derivatives or chemical crosslinking methods, and is the preferred reagent for the preparation of covalent AII–receptor complexes [8].

The bovine adrenal AII–receptor complex behaved as a glycoprotein, and was of almost neutral

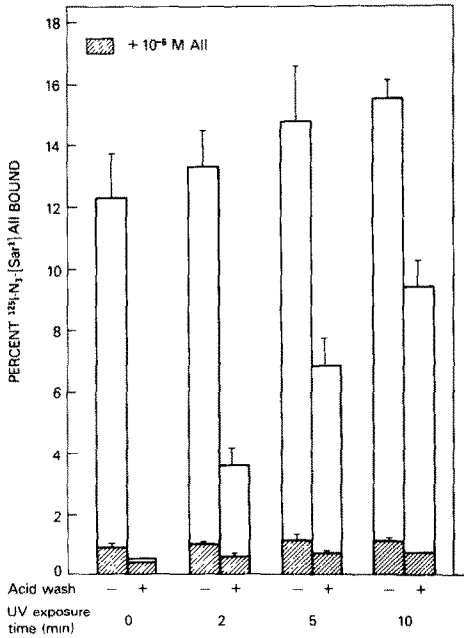

Fig. 1. Covalent labeling of bovine adrenal AII receptors. Membranes were incubated with [^{125}I]N_3–[Sar^1]AII in the absence (clear bars) or presence (hatched bars) of 1 μM AII, then irradiated for varying periods of time, treated with acid as indicated, and washed by filtration to remove free hormone. Bars indicate the mean ± SE of triplicate determinations.

Table 1. Molecular weights of AII binding proteins from different species

	$M_r \times 10^{-3}$			
Tissue	Rat	Rabbit	Monkey	Cow
Adrenal cortex	79 ± 6 (8)	73 ± 8 (3)	74 ± 6 (4)	58 ± 5 (4)
Liver	60 ± 7 (9)	60 ± 6 (3)	78 ± 4 (2)	63 ± 5 (2)

Values indicate the mean ± SE of data from the numbers of experiments given in parentheses.

pI value as indicated by chromatofocusing and ion exchange chromatography. The presence of carbohydrate residues was shown by specific absorption to wheat germ lectin and by the effects of glycosidase digestion. The carbohydrate moieties contributed significantly to the apparent M_r of the receptor, and enzymatic deglycosylation decreased the size of the bovine and rat adrenal receptors by 40% and 55% to 35,000 and 34,000, respectively.

The apparent molecular weight of the photolabeled AII receptor site on SDS–PAGE varied significantly between species (Table 1) and also among receptors of target tissues from the same species (Table 2). The variations in size of the AII receptors of different species and target tissues during SDS–PAGE analysis are illustrated in Fig. 2. The molecular weights of the bovine adrenal, rat and rabbit liver sites were in agreement with those previously reported [3, 6, 8], but the M_r of the rat adrenal AII receptor (79,000) was significantly smaller than that (116,000) reported by others [4]. It is possible that chemical crosslinking of the particulate hormone–receptor complex may lead to overestimation of the molecular weight of the hormone-binding moiety, presumably by the crosslinking of adjacent proteins to the receptor, or of the receptor subunits. Although the M_r of the major photoaffinity-labeled protein in the bovine adrenal cortex was about 60,000 on HPLC gel permeation chromatography and SDS–PAGE, minor species of higher molecular weight were also detected in the absence of reducing agents. The findings, together with the crosslinking data, again suggest that the AII holoreceptor is a dimer of similar-sized subunits.

The labeled protein in rat adrenal glomerulosa (79 kDa) was physically distinct from that in liver, (60 kDa) and pituitary gland (92 kDa). These results may be relevant to the suggestion that receptors in various AII target tissues differ in their pharmacological properties [9]. Only a single broad band was consistently labeled in each tissue by the photoreactive AII derivative. The broadness of the labeled species observed by SDS gel electrophoresis was consistent with the glycoprotein nature of the AII receptor. In some instances the labeled band appeared to be a doublet, but whether this represented two species of AII binding sites, or was the product of minor receptor degradation, is not certain. Since two types of angiotensin receptor have

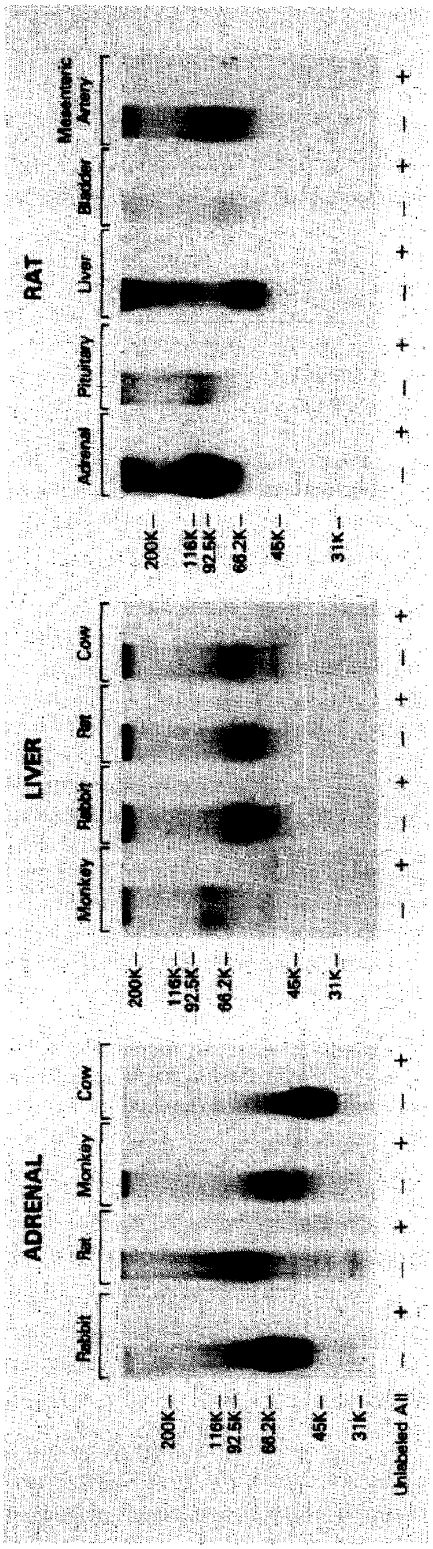

Fig. 2. Labeling of AII receptors from different species and target tissues. Fresh particulate fractions prepared from individual tissues were incubated with either [^{125}I]N_3-[Sar^1]AII or [^{131}I]N_3-[Sar^1]AII in the absence (−) or presence (+) of 1 μM AII. The photolabeled membranes were solubilized and analyzed by SDS–PAGE followed by fluorography.

Table 2. Molecular weights of AII binding proteins from rat tissues

	Adrenal glomerulosa	Liver	Anterior pituitary	Mesenteric artery	Bladder
$M_r \times 10^{-3}$	79 ± 6 (8)	60 ± 7 (9)	92 ± 2 (2)	71 ± 2 (3)	72 ± 3 (2)

Values indicate the mean ± SE of data from the numbers of experiments given in parentheses.

been proposed in cardiac and vascular tissues [10], it is interesting that the labeled bands from smooth muscle tissues (bladder and mesenteric artery) were particularly broad and diffuse.

The basis for the difference between the AII binding sites in rat liver and rat adrenal, and the relevance of their physical properties to functional differences between the two receptors, is not yet clear. There may be more than one gene for the AII receptor, and these could be differentially expressed in the various AII target tissues. However, the more likely possibility is that different post-translational modifications of a single gene product occur within the individual target tissues. Variations in the degree of glycosylation, which contributes substantially to the molecular weights of the AII binding sites, could be responsible for the differences among indivudual target tissues and between species. Thus, the rat adrenal receptor may be simply a more heavily glycosylated form of the corresponding liver receptor, and the protein core of the receptor subunit could be basically similar in all AII target cells.

Role of guanine nucleotide regulatory proteins in aldosterone secretion

The mechanism by which AII elicits aldosterone secretion from the adrenal glomerulosa operates primarily via a calcium-mediated signalling system. Aldosterone stimulation by AII is dependent upon the extracellular Ca^{2+} concentration [11], and is influenced by calcium channel agonists [12] and antagonists [13]. In addition, AII stimulates ^{45}Ca influx [14] and efflux [15], and elevates the cytosolic Ca^{2+} level [16], all of which reflect important aspects of its mechanism of action [17]. AII-induced rises in intracellular Ca^{2+} concentration are believed to be largely the consequence of increased polyphosphoinositide breakdown [17, 18], but the manner in which AII initiates this response, or stimulates Ca^{2+} fluxes, is not yet clear.

Although the putative signal transducing proteins associated with the adrenal plasma membrane AII receptor have not been identified, a potential candidate is the guanine nucleotide regulatory protein, N_i. This protein is functionally coupled to the receptors of several physiological agonists that, like AII [19–21], inhibit adenylate cyclase [22]. Although such agonists have diverse effects in their target tissues, N_i has been generally considered to mediate only that portion of the receptor signal that leads to inhibition of adenylate cyclase, and to act as the inhibitory counterpart to N_s. Studies of N_i have relied extensively on the use of *Bordetella pertussis* toxin (PT), which catalyzes the ADP-ribosylation of the 41,000 dalton α-subunit of N_i in a number of tissues [23].

More recently, several biochemical responses to hormone action not linked to cAMP have been found to be prevented by PT. These responses represent putative intracellular signalling steps associated with hormones with physiological actions that are believed to be predominantly calcium mediated. They include increases in ^{45}Ca influx [24], stimulation [25, 26] and inhibition [27] of phospholipid breakdown, and enhanced release of arachidonic acid [24, 28], as well as decreases in intracellular Ca^{2+} levels [29]. Physiological agonists that have been linked to N_i in this manner include somatostatin [29], FMLP [25, 28], thrombin [24], angiotensin II [24], dopamine [27], and platelet activating factor [24]. Although transduction of inhibitory regulation of adenylate cyclase by N_i seems to be a general phenomenon, in certain tissues N_i also mediates stimulatory responses that are not linked to cAMP. For example, polyphosphoinositide breakdown caused by FMLP in neutrophils is sensitive to PT [22, 25], while stimulation of the same response by thrombin in 3T3 cells is completely unaffected by toxin pretreatment [24]. The apparent tissue specificity and wide range of biochemical responses attributed to N_i may reflect the presence of multiple forms of N_i in various tissues.

In the course of identifying post-receptor mediators of AII action, we examined the influence of PT on several aspects of glomerulosa cell function. The adrenal glomerulosa also contains SRIF receptors [30] that mediate specific inhibition of AII-stimulated aldosterone secretion [31], and provide a positive control for the efficacy of PT treatment upon a defined N_i-linked receptor system in the glomerulosa cell. In rat glomerulosa cells, AII and SRIF attenuated the cyclic AMP response to ACTH by about 40% [32]. These inhibitory effects were prevented by treatment with PT, consistent with their mediation by N_i (Fig. 3). The minor inhibitory effect of AII on basal cAMP levels was also diminished by toxin treatment. In addition, the inhibitory effect of SRIF upon AII-induced aldosterone production was blocked by PT in a dose-dependent manner, again consistent with coupling of the SRIF receptor to an N_i-like regulatory protein. Despite these well-marked effects of PT on N_i-mediated responses, there was no effect of

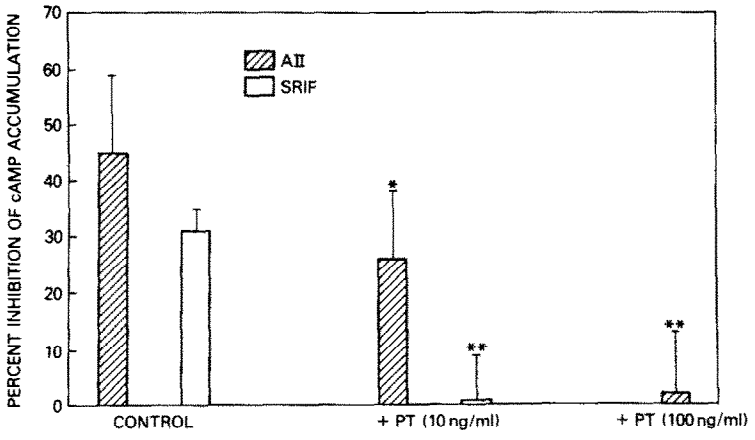

Fig. 3. Effects of PT on inhibition of cAMP production by AII and SRIF. Glomerulosa cells were preincubated for 2 h with the indicated concentrations of PT, then resuspended in medium 199 containing 4.0 mM K^+, 1 mM IBMX, and incubated for 5–10 min with 100 mM AII (hatched bars) or 100 nM SRIF (clear bars), and then for an additional 10–15 min with 10 nM ACTH. Results represent the mean ± SD of data obtained from 4 separate cell preparations, each performed in quadruplicate. ACTH stimulation was 2.6 ± 0.4-fold over the basal cAMP levels, which were 0.98 ± 0.20 pmol/10^5 cells. In control cells, stimulation of cAMP by ACTH was unaffected by the presence of PT. *, $P < 05$; **$P < 0.01$.

toxin pretreatment on the stimulation of aldosterone production by AII.

Since PT influences the binding properties of receptors coupled to N_i, the effects of the toxin on radioligand binding to AII and SRIF receptors and its modulation by guanyl nucleotides were examined. PT treatment had no influence upon AII binding affinity, or on the effect of guanyl nucleotides on AII binding, as shown in Fig. 4. However, in the same cell preparation, PT almost completely abolished [^{125}I]Tyr^0-SRIF binding and its modulation by guanyl nucleotides. The toxin was also without effect on AII-stimulated phosphoinositide breakdown in intact glomerulosa cells, and on GTPase activity in permeabilized cells, again consistent with predominant coupling of the AII receptor to a nucleotide regulatory protein other than N_i. ADP-ribosylation studies with PT revealed a single 41 kDa band in adrenal membranes, which was completely ADP-ribosylated under conditions in which neither AII binding or AII-stimulated GTPase activity were influenced by toxin treatment.

While no indication was found for a role of N_i in the stimulatory actions of angiotensin II, the evidence from cAMP studies that the AII receptor can also couple to N_i prompted further analysis of the action of PT over a wide range of AII concentrations. In several species, AII has an inhibitory effect on aldosterone production when present at supramaximal concentrations. As shown in Fig. 5, such attenuation of the aldesterone response by high AII concentrations was abolished by pretreatment of glomerulosa cells with PT.

These observations suggest that a functional N_i

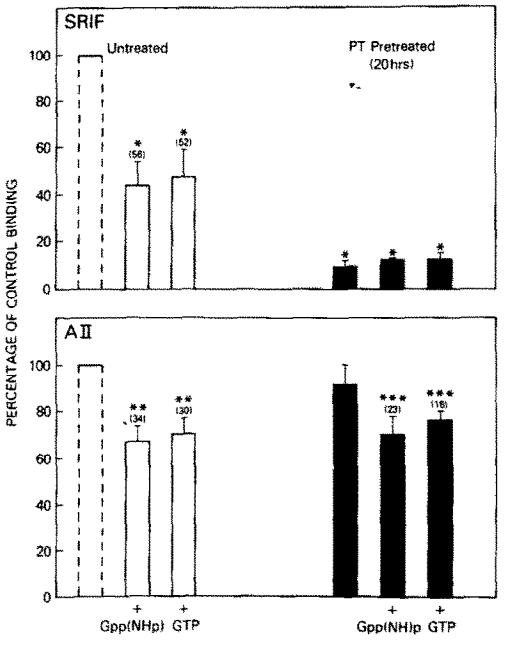

Fig. 4. Effects of PT on hormone binding to rat glomerulosa cells. Glomerulosa cells were preincubated for 20 h with (dark bars) or without (clear bars) 1 μg/ml PT, centrifuged, and vigorously resuspended in hypotonic buffer prior to binding assays with radioiodinated SRIF (above) or AII (below). Data are expressed as the mean ± SD of assays of triplicate samples in 3 or 4 separate experiments. Control binding was 2770 ± 500 cpm/tube for [^{125}I]Tyr^0-SRIF, and 3750 ± 700 cpm/tube for [^{125}I]AII. Asterisks indicate significant differences from control [^{125}I]Tyr^0-SRIF binding (*, $P < 0.01$); from control [^{125}I]AII binding (**, $P < 0.05$); and from PT-pretreated control [^{125}I]AII binding (***, $P < 0.05$).

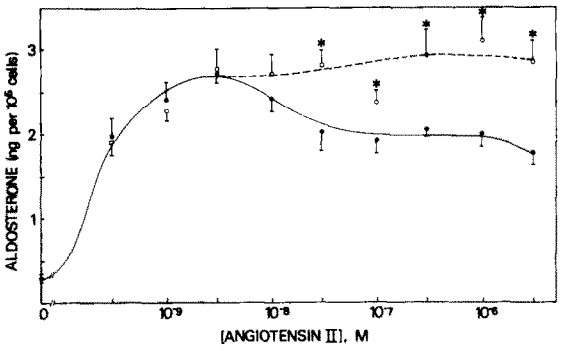

Fig. 5. Blockade of supramaximal AII inhibition of aldosterone production by PT. Glomerulosa cells were preincubated with (open circles) or without (closed circles) 100 ng/ml PT for 2 h in the presence of increasing concentrations of AII. *, $P < 0.05$.

protein in the adrenal glomerulosa can be coupled to both AII and SRIF receptors. This is consistent with recent evidence that AII receptors in the liver [33] and the pituitary [27], like SRIF receptors in several tissues [34], are functionally linked to N_i as indicated by the sensitivity of adenylate cyclase inhibition to PT treatment. However, it is clear that N_i does not mediate the stimulatory actions of AII on aldosterone production. The mechanism of action of the N_i-like protein on the inhibition of the steroid response remains uncertain. It is possible that the PT-sensitive component is related to an effect on cAMP, although AII stimulation of aldosterone production does not involve elevation of cAMP formation [35].

These results in the adrenal cortex are analogous to recent observations in the anterior pituitary [27], where the toxin did not impair prolactin secretion stimulated by AII, despite inhibition of the negative effects of AII on adenylate cyclase. In hepatocytes, PT had no effect on the stimulation of phosphorylase activity by AII [36]. Also N_i-like proteins can mediate some but not all cellular responses to other hormones, as observed in mouse 3T3 cells with thrombin and AII as agonists [30], and in chick heart cells with carbachol as agonist [37].

Both the GTPase and the binding studies suggest that an additional guanine nucleotide binding protein in the adrenal cortex is coupled to the AII receptor. Although it might be expected that at least a portion of this GTP-mediated binding effect would reflect the coupling of AII receptors to an N_i-like protein, and would thus be abolished by toxin pretreatment as seen in the liver [36], we did not observe any effect of PT on AII binding. The nature and function of the second N protein in the glomerulosa cell remain to be clarified, but recent observations suggest that additional guanine nucleotide-binding proteins are involved in receptor coupling to phospholipase C. Guanine nucleotides have been found to perturb carbachol binding in 1321N1 cells [38], α_1-adrenergic binding in the renal cortex [39], and TRH binding to GH_4C_1 pituitary cells [29], in a PT-insensitive manner. Each of these hormones, like AII, promotes the breakdown of polyphosphoinositides. Also, several studies in membrane systems and permeabilized cells have shown facilitation of polyphosphoinositide turnover by guanine nucleotides [40–43]. It is likely that the second N protein in the adrenal cortex (N_x) mediates the phospholipid response to AII, and the subsequent stimulation of aldesterone secretion.

In the absence of firm evidence of receptor subtypes in the adrenal cortex, these observations demonstrate that stimulation of a cellular response by a single agonist may, in some cases, be mediated by coupling of the receptor to more than one N protein. These results also provide an explanation for the dose-dependence of the "desensitization of the steroid response seen in glomerulosa cells with AII, namely that at sufficiently high concentrations the hormone–receptor complex may interact in a low-affinity manner with the N_i-like protein which can then inhibit aldosterone production.

AII Receptors and phosphoinositide hydrolysis

The action of angiotensin II upon aldosterone production in the adrenal gland is exerted through receptor-mediated hydrolysis of inositol phospholipids [44, 45] and increased cytosolic calcium concentration [16, 17, 46]. Such ligand-induced turnover of phosphoinositides is a common feature of the trans-membrane signalling mechanism during activation of target cells by calcium-dependent hormones and other stimuli [47]. The most important event in this process is the cleavage by phospholipase C of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) to provide inositol-1,4,5-trisphosphate (Ins-1,4,5-P_3) and sn-1,2-diacylglycerol, both of which act as intracellular second messengers to mediate hormone action [47, 48]. The ability of Ins-1,4,5-P_3 to release Ca^{2+} from a non-mitochondrial compartment, presumably the endoplasmic reticulum [49], is probably a major determinant of rapid increases in cytoplasmic Ca^{2+} concentration. Since the concomitant product of PtdIns-4,5-P_2 hydrolysis, diacylglycerol, is a potent activator of protein kinase C [48], temporal or quantitative interactions between the two branches of the calcium messenger system have been proposed [17, 48].

Recent studies on the metabolism of phosphoinositides have revealed new aspects of inositol polyphosphate formation and metabolism in ligand-activation cells. The first indication of a more complex route of inositol phosphate metabolism was the finding of another isomer of inositol trisphosphate, identified as Ins-1,3,4-P_3, during cholinergic stimulation of the parotid gland [50]. This isomer, in addition to the biologically active product, Ins-1,4,5-P_3, is also present in hepatocytes and DMSO-differentiated HL-60 cells [51]. The origin of the

1,3,4-isomer was originally uncertain, but recent evidence has shown it to be produced from Ins-1,4,5-P_3 through conversion to Ins-1,3,4,5-P_4 and subsequent 5-dephosphorylation [52, 53].

During studies on the stimulation of aldosterone production by angiotensin II, the early events of phosphoinositide metabolism were analyzed in adrenal zona glomerulosa cells [54]. For this purpose, rat glomerulosa cells were labeled with myo-[^{3}H]inositol for 3 h prior to stimulation with AII in the absence or presence of 10 mM LiCl. Incubations were terminated by addition of ice-cold TCA and cells were frozen prior to extraction of the supernatant with dietyl ether and fractionation of inositol phosphates by HPLC on a strong ion exchange column with elution by 0–0.7 M ammonium phosphate, pH 3.35.

The typical HPLC elution profiles of one control and two stimulated samples obtained from adrenal glomerulosa cells prelabeled with [^{3}H]inositol are shown in Fig. 6. Of particular interest was the presence of two isomers of inositol monophosphate, the first eluted being Ins-1-P and the second identified as Ins-4-P. Two forms of Ins-P_3 were also distinguished, the 1,4,5-isomer and an immediately preceding peak that was present only in extracts from hormone-stimulated cells. Based on its slightly shorter retention time and the co-migration with [^{3}H]ATPstandard, this peak was identified as Ins-1,3,4-P_3 [50]. Extended gradient elution did not reveal additional peaks corresponding to higher inositol phosphates, either in control or angiotensin-stimulated glomerulosa cells.

Addition of angiotensin II was followed immediately by a marked increase in the formation of Ins-1,4-P_2, which was associated with only a slight and transient elevation of Ins-1,4,5-P_3, and the appearance of a peak of Ins-1,3,4-P_3. The most prominent feature of the early inositol phosphate response was the rapid increases of Ins-4-P in the absence of a change in Ins-1-P, an expected product of inositol polyphosphate metabolism. Since Ins-4-P can be derived only from the hydrolytic products of polyphosphoinositides [55], its marked increase

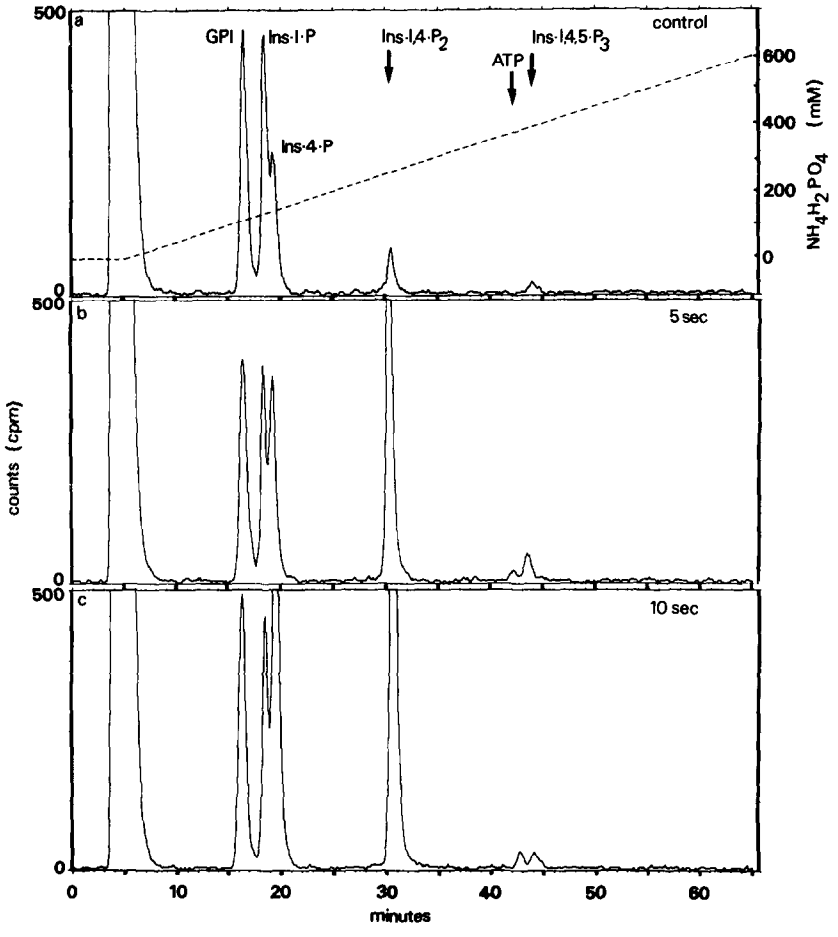

Fig. 6. Anion exchange HPLC elution profile of inositol phosphates from control and AII-stimulated rat glomerulosa cells. Cells were prelabeled with [^{3}H]inositol for 3 h and stimulated with 10^{-8} M AII for 5 s (panel b) or 10 s (panel c). The gradient of $NH_4H_2PO_4$ used for elution and the elution positions of reference standards are shown in the upper panel. ATP indicates the position of Ins-1,3,4-P_3; GPI: glycerophosphoinositol.

within 5 s after angiotensin stimulation indicates the extreme rapidity of formation and degradation of inositol polyphosphates during AII action.

Although both Ins-1,4,5-P_3 and Ins-1,3,4-P_3 increased rapidly during AII stimulation, the change in Ins-1,4,5-P_3 was transient, with a 2-fold rise at about 5 s (Fig. 7). On the other hand, Ins-1,3,4-P_3 increased progressively during the first 60 s and remained elevated for the 20 min period. In view of the small amount of Ins-1,4,5-P_3 detected by HPLC during stimulation by angiotensin II, the breakdown of PtdIns-4,5-P_2 and PtdIns-4-P was also determined in each experiment. As anticipated, there was rapid and substantial hydrolysis of both polyphosphoinositide precursors in angiotensin II-stimulated cells during the first 60 s of incubation.

The minor changes in the biologically active isomer, Ins-1,4,5-P_3, contrasted with the extremely large increase in the level of its metabolite, Ins-1,4-P_2. The peak increase of almost 10-fold in Ins-1,4-P_2 occurred at 10 s and was followed by a decline to an elevated steady-state level that was maintained throughout the 20 min incubation period (not shown). Very rapid changes in the level of Ins-4-P, but not of Ins-1-P, were observed in the first minute of stimulation. The increase in Ins-4-P was already apparent at 2.5 s and reached its maximum at about 30 s, followed by a steady-state high level throughout the 20 min period. In contrast, Ins-1-P was moderately increased only after 2.5 min of stimulation by angiotensin II (Fig. 8).

When cells were stimulated with angiotensin II in the presence of lithium, only the 1,3,4-isomer of inositol trisphosphate continued to accumulate with time. In contrast to the major increase in Ins-1,3,4-P_3, there was no significant effect of lithium on the levels of Ins-1,4,5-P_3 during hormonal stimulation (Fig. 7). The basal level of Ins-1,4-P_2 was only slightly increased (about 2-fold) in the presence of Li^+, but stimulation with angiotensin II caused a very rapid increase and continuous accumulation of this metabolite (not shown). Lithium caused major elevations in the basal levels of both isomers of inositol monophosphate. Against this high basal activity, changes in Ins-4-P during angiotensin stimulation were not apparent for up to 30 s, but thereafter Ins-4-P increased progressively to extremely high levels throughout the 20 min period. Accumulation of Ins-1-P was also prominent in lithium-treated cells, and although its increase was delayed by comparison with the 4-monophosphate isomer, the levels of the two monophosphates were similar

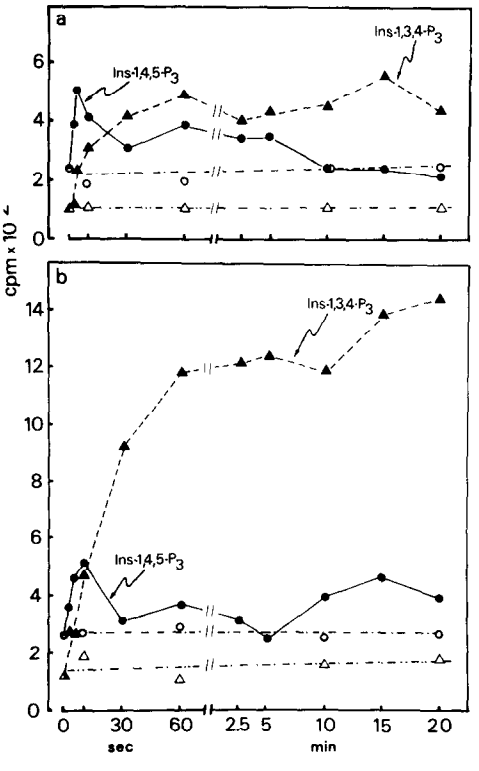

Fig. 7. Formation of Ins-1,4,5-P_3 (—○—) and of Ins-1,3,4-P_3 (—△—) in AII-stimulated vs control (—○—, —△—) rat glomerulosa cells. Cells were prelabeled with [^{3}H]inositol and incubated without (a) or with (b) 10 mM LiCl for 40 min. Angiotensin II (10^{-8} M) was then added for the times indicated and the inositol phosphates were analyzed by HPLC.

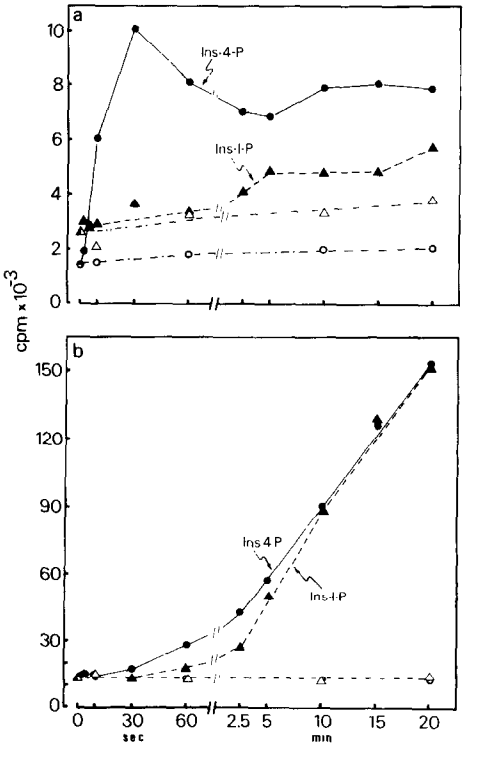

Fig. 8. Formation of Ins-4-P (—○—) and Ins-1-P (—△—) in AII-stimulated vs control (—○—, —△—) rat glomerulosa cells. Panel a: no LiCl, Panel b: 10 mM LiCl. Note the difference in the scales of the ordinates in Panels a and b.

between 5 and 20 min of stimulation by angiotensin II (Fig. 8).

Following its generation by phospholipase C-catalyzed breakdown of PtdIns-4,5-P_2, the active 1,4,5-isomer of Ins-P_3 binds to high-affinity intracellular receptors [56, 57] to mobilize stored Ca^{2+}, and is rapidly degraded by specific phosphatases to Ins-1,4-P_2, inositol monophosphates and finally inositol [58,59]. However, Ins-1,4-P_2 and Ins-1-P could also be produced by the actions of phospholipase C on PtdIns-4-P and PtdIns, respectively. Thus, it may be difficult to decide whether changes in the levels of these metabolites solely reflect degradation of Ins-1,4,5-P_3, or whether phospholipase C activity is not restricted to PtdIns-4,5-P_2.

It is clear that AII stimulation causes extremely rapid changes in the levels of all inositol phosphates in adrenal glomerulosa cells. There was no significant time delay in the appearance of Ins-1,4-P_2 and Ins-1,3,4-P_3 when compared to the increase in Ins-1,4,5-P_3, and even Ins-4-P was significantly increased at the earliest time point (2.5 s) of stimullation. In contrast to the extremely large increases of its putative metabolites, the elevation of Ins-1,4,5-P_3 never exceeded 3-fold. Comparison of the radioactivities of the several inositol phosphates indicates that large amounts of Ins-1,4,5-P_3 must be produced and converted to Ins-1,4-P_2 and Ins-4-P in the first few seconds of stimulation. The rapid and substantial decrease of PtdIns-4,5-P_2 also suggest that considerable amounts of Ins-1,4,5-P_3 had been generated and served as the major source of the high levels of Ins-1,4-P_2. However, the concomitant and less marked decrease of PtdIns-4-P could reflect its hydrolysis by phospholipase C to form Ins-1,4-P_2, as well as its rapid conversion to PtdIns-4,5-P_2.

The rapid elevation of Ins-4-P in the absence of a significant change of Ins-1-P during the first minute of stimulation suggests that sequential conversion to Ins-1,4-P_2 and Ins-4-P is the preferred pathway for Ins-1,4,5-P_3 degradation in the glomerulosa cell. As further implication of these results is that PtdIns is not cleaved by phospholipase C during the first minute of stimulation by angiotensin II. Whether the subsequent increase in Ins-1-P formation after 2.5 min reflects the direct breakdown of PtdIns is not yet certain. In angiotensin-stimulated vascular smooth muscle cells, biphasic production of diacylglycerol has been attributed to a shift in phospholipase C activity from polyphosphoinositides toward PtdIns [60]. The delayed increase in the level of Ins-1-P in the present study is compatible with such a "late" activation of PtdIns hydrolysis, but the alternative route of its production via degradation of the rising amounts of Ins-1,4-P_2 can not be excluded.

The inhibition of inositol-1-phosphatase by lithium [61] has been widely used as a maneuver to amplify ligand-induced changes in inositol phosphate production. In AII-stimulated cells, both Ins-4-P and Ins-1-P accumulated in the presence of lithium, suggesting that dephosphorylation of both inositol monophosphates is inhibited. In human platelets and rat gonadotrophs, accumulation of Ins-4-P was augmented by Li^+ during stimulation by vasopressin [62] and GnRH [55], respectively. The progressive accumulation of Ins-4-P in the presence of Li^+ indicates that polyphosphoinositides are continuously degraded during angiotensin II action. There was little if any effect of Li^+ on the level of Ins-1,4,5-P_3 in AII-stimulated glomerulosa cells. The minor and transient rise in Ins-1,4,5-P_3, in association with the high level of Ins-1,4-P_2, indicates that removal of the active calcium-mobilizing isomer must be both rapid and highly effective. Such a mechanism may serve to terminate the initiation of the biological response, and also to ensure that the intracellular milieu is only briefly exposed to elevated cytosolic calcium concentrations.

It is clear that, as in pancreatic acinar cells [51], accumulation of Ins-1,3,4-P_3 rather than the biologically active 1,4,5 isomer is responsible for the increased levels of inositol trisphosphate observed in many tissues when stimulated in the presence of Li^+ and analyzed by Dowex ion exchange chromatography. The immediate appearance of Ins-1,3,4-P_3, together with the absence of detectable higher inositol phosphates, indicate that Ins-1,3,4,5-P_4, the intermediate in the conversion of Ins-1,4,5-P_3 to Ins-1,3,4-P_3 [52, 53], must be formed extremely rapidly and in amounts below the level of detection in inositol-labeled rat glomerulosa cells.

To extend these observations, the actions of AII on inositol polyphosphate formation and metabolism were analyzed in cultured bovine glomerulosa cells after labeling with [^{3}H]inositol for 24 h [63]. In this system, AII stimulated the production of several inositol phosphates, as shown in Fig. 9. The most prominent effect of AII is the substantial increase in two isomers of InsP_2, one corresponding to Ins-1,4-P_2 and another that probably represents Ins-1,3-P_2 or Ins-3,4-P_2. The production of two isomers of Ins-P (Ins-1-P and Ins-4-P) is also substantially increased, especially that of Ins-4-P. The production of Ins-P_3 was increased by 4-fold after 5 min of stimulation. A detailed analysis of the Ins-P_3 peak shown in Fig. 9 (inset) revealed that it consisted of two isomers, the amount of Ins-1,3,4-P_3 far exceeding that of Ins-1,4,5-P_3. A finding of major interest was the consistent production of a metabolite with the elution properties of inositol tetrakisphosphate. This compound increased in a time-dependent fashion upon AII stimulation, rising from undetectable levels and in proportion with the increase in Ins-P_3. In contrast, a more highly acidic compound with elution properties corresponding to Ins-P_5 did not show any significant change during hormonal stimulation.

In order to ascertain the identity of Ins-P_4 and to

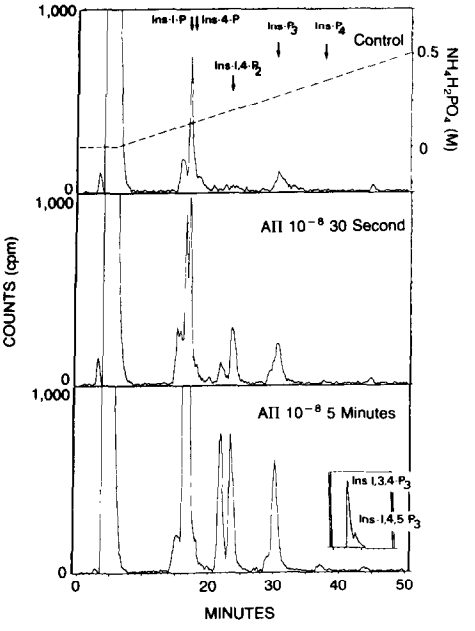

Fig. 9. HPLC analysis of inositol phosphates formed in AII-stimulated bovine glomerulosa cells. After pre-labeling for 24 h with [^{3}H]inositol, cells were stimulated with 10 mM AII, for 30 s (middle panel) and 5 min (lower panel). Arrows in the upper panel (control) indicate the elution times of radioactive standards.

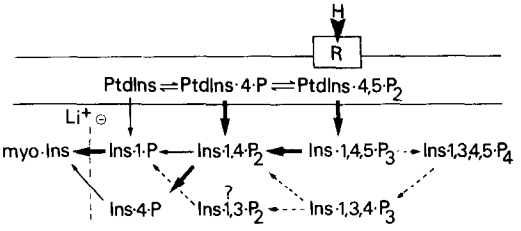

Fig. 10. Production and metabolism of inositol phosphates during activation of phospholipase C angiotensin II in adrenal glomerulosa cells. Similar metabolic pathways are involved in the disposition of inositol phosphates formed during hormone–receptor interactions in many calcium-dependent target cells. Bold arrows show major pathways of production or elimination of the individual metabolites, and dashed arrows indicated recently-defined pathways of inositol polyphosphate metabolism.

verify the presence of the enzyme responsible for its production in the bovine adrenal cortex, adrenal cytosol was assayed for Ins-P_3-kinase activity [53]. The partially purified enzyme preparation actively converted [^{3}H]Ins-P_3 to [^{3}H]Ins-P_4, which coeluted during HPLC with the Ins-P_4 endogeneously produced by AII-stimulated glomerulosa cells, confirming the identity of the latter metabolite.

The multiplicity of inositol polyphosphates and isomers detected by anion exchange HPLC during AII stimulation indicates the complexity of the metabolism of Ins-1,4,5-P_3 following its hormone-activated cleavage from phosphatidylinositol bisphosphate in the plasma membrane (Fig. 10). The generation of two isomers of Ins-P, Ins-P_2, and Ins-P_3, as well as the presence of Ins-P_4 and Ins-P_5, extend the above observations on inositol phosphate production by rat glomerulosa cells. The preferential formation of Ins-4-P indicates that stimulated polyphosphoinositide catabolism proceeds mainly via the 4-monophosphate metabolite, and also that direct hydrolysis of phosphatidylinositol is not an early response to hormone stimulation.

Some of these results differ from those obtained with rat glomerulosa cells labeled for 3 h with [^{3}H]inositol, in which AII-stimulated increases in the levels of both isomers of Ins-P_3 were relatively small and were not associated with detectable production of Ins-P_4. In cultured bovine glomerulosa cells labeled with [^{3}H]inositol for 24h, AII-induced formation of Ins-P_3 was much more prominent and was associated with the appearance of a compound that co-eluted with standard Ins-P_4. This difference may be related to the lengths of the labeling periods or to a species difference in the rates of production and/or degradation of Ins-P_3 and Ins-P_4. In bovine adrenal cells, the presence of a cytosolic kinase able to phosphorylate Ins-1,4,5-P_3, together with the AII-stimulated formation of Ins-1,4,5-P_3 and its higher phosphorylated derivative (Ins-1,3,4,5-P_4) and the formation of a second Ins-P_3 isomer (Ins-1,3,4-P_3) presumably derived from 5′-dephosphorylation of InsP_4, provides direct evidence for the existence of the inositol Tris–tetrakisphosphate pathway. The presence of such a pathway was also suggested by the recent observation of an Ins-P_3 kinase activity in permeabilized adrenal cells [64].

The functional significance of this pathway of Ins-P_3 metabolism is not yet clear: it might represent a second route (together with the 5′-phosphatase) for inactivation of Ins-1,4,5-P_3, or it may generate a second messenger that is responsible for one of the steps involved in stimulation of steroidogenesis. The presence of a new isomer of Ins-P_2, which may be either Ins-1,3-P_2 or Ins-3,4-P_2, is an additional feature of interest, since it may represent a specific metabolite of Ins-1,3,4-P_3. The detailed analysis of the rates of appearance and disappearance of the multiple inositol phosphates and isomers generated during AII stimulation should provide a better understanding of the metabolic pathways and roles of such compounds in the hormone-stimulated glomerulosa cell.

Intracellular receptors for IP_3 in angiotensin II target tissues

Following the cleavage of inositol phospholipids by phospholipase C during the response to stimulation by AII and other regulatory ligands, Ins-P_3 and diacylglycerol are believed to initiate target-cell respones by increasing the intracellular calcium concentration [47] and by activating protein kinase C [48], respectively. InsP_3 releases sequestered Ca^{2+} from an intracellular store that is distinct from the

mitochondria [47, 65], and is believed to interact with a specific receptor on the endoplasmic reticulum. Indirect evidence that $InsP_3$ binds to a saturable intracellular receptor was provided by studies on the inhibition of InP_3-induced Ca^{2+} release in macrophages by prior blockade of the putative binding sites with a photoaffinity derivative of $InsP_3$ [66]. In addition, we have recently demonstrated that high-affinity and saturable $InsP_3$ binding sites are present in homogenates of the bovine adrenal cortex [56]. Similar $InsP_3$ binding sites have also been identified in permeabilized neutrophils and hepatocytes, and in a liver microsomal fraction [57, 67]. The $InsP_3$ receptor has been further characterized in the bovine adrenal cortex and other target tissues [68]. In the adrenal cortex, $InsP_3$ binding sites are saturable and present in low concentration (104 ± 48 fmol/mg protein), and exhibit high-affinity for inositol 1,4,5-trisphosphate (K_d 1.7 ± 0.6 nM). Their ligand specificity is illustrated by their low-affinity for inositol 1,4-bisphosphate ($K_d \sim 10^{-7}$ M), inositol 1-phosphate and phytic acid ($K_d \sim 10^{-4}$ M), fructose 1,6-bisphosphate and 2,3-bisphosphoglycerate ($K_d \sim 10^{-3}$ M), with no detectable affinity for inositol 1-phosphate and myo-inositol. These binding sites are distinct from the degradative enzyme, isositol trisphosphate phosphatase, which has a much lower affinity for inositol trisphosphate ($K_m = 17$ μM). Furthermore, submicromolar concentrations of inositol 1,4,5-trisphosphate evoked a rapid release of Ca^{2+} from non-mitochondrial ATP-dependent storage sites in the adrenal cortex. Specific and saturable binding sites for inositol 1,4,5-trisphosphate were also observed in the anterior pituitary ($K_d = 0.87 \pm$ 0.31 nM; 14.8 ± 9.0 fmol/mg protein) and in the liver ($k_d = 1.66 \pm 0.07$ nM; 147 ± 24 fmol/mg protein. These observations suggest that the specific $InsP_3$ binding sites in adrenal, liver and pituitary represent the functional receptors through which inositol 1,4,5-trisphosphate mobilizes Ca^{2+} in target tissues for angiotensin II and other calcium-dependent hormones.

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