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STUDIES OF HORMONE-SENSITIVE AND -INSENSITIVE POOLS OF PHOSPHOINOSITIDES IN CULTURED BOVINE ZONA FASCICULATA/RETICULARIS CELLS

EVIDENCE THAT ACETYLCHOLINE AND ANGIOTENSIN II STIMULATE THE BREAKDOWN OF A COMMON POOL OF PHOSPHOINOSITIDES

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Abstract—The effects of acetylcholine (ACh) and manganese pre-incubation on angiotensin II (AII)-stimulated incorporation of [^{3}H]inositol into phosphoinositide, phosphoinositol and free inositol fractions of adrenocortical cells isolated from the bovine zona fasciculata/reticularis (zfr) were investigated. In cells pre-labelled for 6 hr with [^{3}H]inositol, ACh and AII stimulated the incorporation of cytosolic [^{3}H]inositol into a common hormone-sensitive pool of phosphoinositides, which was distinct from the non-hormone-sensitive pool labelled in the presence of manganese. Regression analysis of the cortisol versus [^{3}H]inositol headgroup responses for both AII (10^{-11}–10^{-7} M) and ACh (10^{-9}–10^{-3} M) showed that the gradients of these responses were not significantly different. These data provide strong evidence that in cultured bovine zfr cells, ACh and AII stimulate the breakdown and resynthesis of a common pool of phosphoinositides.

Contrary to the earlier view that adrenocorticotrophin (ACTH§) is the sole stimulus of steroid secretion from the inner zones of the adrenal cortex, it is now clear that zona fasciculata/reticularis (zfr) cells secrete steroids in response to a wide variety of hormones and neurotransmitters. Studies by ourselves and others have established that bovine zfr cells, either freshly isolated or in primary culture, secrete cortisol in response to catecholamines [1, 2], angiotensin II (AII) [2, 3], acetylcholine (ACh) [2, 4, 5] and vasopressin [6] in addition to ACTH. Of these agonists, AII and ACh have been shown to exert this effect through the activation of a hormone-sensitive phosphoinositidase C [6, 7]. Neither agonist has any acute effect on cAMP levels, whereas both promote the rapid and dose-dependent formation of water-soluble [^{3}H]phosphoinositols. The phosphoinositols formed and their time-course of appearance are consistent with the activation of a phosphoinositide-specific phosphoinositidase C. In fura-2-loaded zfr cell suspensions, both AII and ACh stimulate dose-dependent increases in cytosolic [Ca^{2+}], and both agonists appear to mobilize a common intracellular Ca^{2+} pool [8]. Furthermore, in single fura-2-loaded bovine adrenocortical cells, oscillations in intracellular [Ca^{2+}] have been observed in response to both agonists [9, 10].

It is also clear that individual cells can respond to both AII and ACh as both agonists mobilize a common intracellular Ca^{2+} pool. It is not clear, however, whether ACh and AII receptors are coupled to phosphoinositidase C which acts on separate subpools of phosphoinositides, or whether phosphoinositidase C acts on a common pool of phosphoinositides. This question is particularly relevant given the evidence for the existence of multiple pools of phosphoinositides in these and other cells [11–13].

In this paper we present evidence that AII and ACh act to stimulate both the breakdown and resynthesis of a common pool of phosphoinositides.

MATERIALS AND METHODS

Materials. Ham's F10 growth medium, Earl's balanced salt solution, glutamine and other cell culture materials were from Northumbria Biologicals (Cramlington, U.K.). Penicillin, streptomycin and fungizone were obtained from Flow Laboratories (Rickmansworth, U.K.) and collagenase from Lorne Diagnostics (Bury St. Edmunds, U.K.). Controlled Process Serum Replacement No. 5, glucose, ACh, atropine, inositol and cortisol were purchased from the Sigma Chemical Co. (Poole, U.K.) and bovine serum albumin (fraction V) was obtained from ICN Biomedical (High Wycombe, U.K.). AII (Asp^1-Val^5) was the MRC international standard from the National Institute for Biological Standards and Control. *myo*[^{3}H]Inositol and cortisol tracer [cortisol-3-(*O*-carboxymethyl) oximino-(2-[^{125}I]iodohistamine)] were supplied by Amersham Inter-

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§ Abbreviations: zfr, zona fasciculata/reticularis; AII, angiotensin II; ACh, acetylcholine; ACTH, adrenocorticotrophin; GM, growth medium; EBSBG, Earl's balanced salt solution containing bovine serum albumin (0.2%) and glucose (0.1%).

national (Amersham, U.K.). 1,1,2-Trichlorotrifluoro-ethane and tri-*n*-octlyamine were purchased from the Aldrich Chemical Co. (Gillingham, U.K.), and all other chemicals to Analar grade from BDH (Thornlibank, Glasgow, U.K.). AG1X8 anion exchange resin (100–200 mesh, formate form) was obtained from Bio Rad Laboratories (Watford, U.K.). Scintillation fluid (299) and scintillation vials were purchased from Canberra Packard (Pangbourne, U.K.), and scintillation counting was carried out on a Canberra Packard 1900CA liquid scintillation counter.

Cell culture. Bovine adrenal glands were obtained from freshly slaughtered 1–2-year-old steers at the local abattoir, and purified zfr cell suspensions prepared as described previously [3]. Cells were plated out at a density of 333,000 cells/well in multiwell plates (12 × 25-mm diameter wells) in 1 mL/well Ham's F10 medium supplemented with 10% Controlled Process Serum Replacement No. 5, penicillin (50 IU/mL), streptomycin (50 μg/mL) and amphotericin B (2.5 μg/mL) (growth medium, GM). GM was renewed after 24 hr (0.5 mL), and the cells maintained in primary culture for a further 48 hr before use. Labelling of the phosphinositides was achieved by inclusion of [^{3}H]inositol in the GM (10 μCi/mL, 0.5 mL) for the last 6 hr of culture (Figs 1 and 2) or for the entire 48-hr period (Fig. 3) where steady-state labelling was required [7].

Where manganese-stimulated labelling was investigated, $MnCl_2$ (1 mM) was included in the GM during the 6-hr labelling period. Where cells were pre-stimulated with ACh during labelling, 5 μL of a stock solution of ACh (in GM) was added to each well to a final concentration of 10^{-4} M for the last hour of labelling only. Pre-stimulation was terminated by addition of 5 μL of the ACh antagonist atropine (10^{-6} M final concentration) to each well for 5 min.

Agonist stimulation of phosphoinositidase C. All agonists were dissolved in Earl's balanced salt solution containing bovine serum albumin (0.2%) and glucose (0.1%) (EBSBG). After the labelling period, GM was replaced with EBSBG (0.5 mL/well), or EBSBG containing atropine (10^{-6} M) if cells had been stimulated previously with ACh. Cells were incubated for 15 min after which medium was replaced with EBSBG with added LiCl (10 mM) and inositol (10mM) (0.45 mL/well). After a further 15 min incubation, agonist solutions (50 μL) were added to a final volume of 0.5 mL, and stimulation allowed to continue for the times indicated before termination by addition of ice-cold perchloric acid (15% v/v, 250 μL/well).

Recovery and measurement of [^{3}H]*inositol-labelled products.* Following addition of perchloric acid to terminate cell stimulation, the base of each well was scraped using a rubber policeman and the contents transferred to an Eppendorf tube, with a 0.5 mL water wash. Following centrifugation (3300 *g* × 3 min), the acid supernatant was transferred to a glass tube and neutralized by mixing with a 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoro-ethane (1.5 mL). Following a brief centrifugation (20 *g* × 5 min) to separate the phases, 0.9 mL of the upper aqueous phase was recovered and stored at −20° prior to assay.

For assay of [^{3}H]inositol and [^{3}H]phosphoinositols, samples were thawed, EDTA added to a final concentration of 1 mM and samples loaded onto individual columns of AG1X8 anion exchange resin (0.25 mL). The columns were washed with water (2 × 4 mL), and the combined eluates from loading and washing (containing free [^{3}H]inositol) collected into a 20 mL scintillation vial. Bound [^{3}H]inositol phosphates were then eluted directly into a fresh 20-mL vial with 1 M ammonium formate–0.1 M formic acid (2 × 2 mL). Following addition of scintillation fluid (10 mL), radioactivity of the samples was determined by liquid scintillation counting. All values shown are volume corrected, and were counted for 10 min each, or to an error of <1%.

The pelleted material recovered from the centrifuged perchloric acid extracts was frozen at −20° under 200 μL water. After thawing, the loosened pellets were broken up by vortexing and dissolved by addition of $CHCl_3$–MeOH–concnHCl (100:200:1 v/v) (0.75 mL), followed by $CHCl_3$ and HCl (0.1 M) (both 250 μL). The resulting aqueous and organic phases were separated by centrifugation (20 *g* × 5 min) and 400 μL of the lower phosphoinositide-containing phase recovered to a scintillation vial. The solvent was removed under air (Techne sample concentrator), 3 mL scintillation fluid added, and radioactivity determined by scintillation counting.

Agonist stimulation of cortisol secretion. For studies on cortisol secretion from unlabelled cells, cells were washed in EBSBG (2 × 1 mL) and incubated in EBSBG for 5 min (0.45 mL). Following addition of agonists to a final volume of 0.5 mL, cells were incubated for 1 hr after which the overlying medium was removed and stored at −20° prior to cortisol radioimmunoassay [14].

Statistical analysis. Statistical significance between results was assessed using Student's *t*-test. Values are given as the means ± SD, unless otherwise stated.

RESULTS

Figure 1 shows the results of studies carried out on cells pulse-labelled for 6 hr (not to steady state) with [^{3}H]inositol, in the presence or absence of ACh (10^{-4} M) or Mn^{2+} (10^{-3} M) as described, and subsequently challenged with AII (10^{-7} M) (in the presence of lithium) for 15 min. The distribution of radioactivity between phosphoinositide (a) free inositol (b) and total phosphoinositol fractions (c) is shown. Under these labelling conditions the bulk of the radiolabel is found in the free inositol fraction.

In control cells (no pre-stimulation during labelling), subsequent acute (15 min) stimulation with AII increased the turnover of the phosphoinositides as indicated by increased accumulation of label in both the phosphoinositides (a) and phosphoinositols (c), with a corresponding decrease in free [^{3}H]inositol (b) (all $P < 0.01$). Although labelling in the presence of ACh increased the incorporation of [^{3}H]inositol into the phosphoinositides ($P < 0.05$), the maximal incorporation

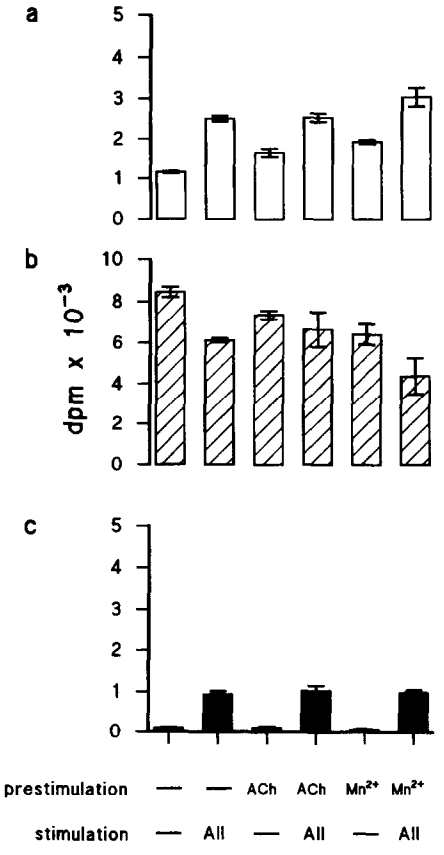

Fig. 1. Effect of ACh and Mn^{2+} pre-stimulation on subsequent acute AII-stimulated redistribution of radioactivity between [^{3}H]phosphoinositide, [^{3}H]inositol and [^{3}H]phosphoinositol fractions of pre-labelled bovine zfr cells. Cells were pre-labelled for 6 hr with [^{3}H]inositol in the presence or absence of MnCl (10^{-3} M) or ACh (10^{-4} M), as described. Cells were subsequently incubated for a further 15 min in the presence or absence of AII (10^{-7} M) in fresh medium containing 10 mM Li^+. The distribution of radioactivity between the [^{3}H]-phosphoinositide (panel a), [^{3}H]inositol (panel b) and [^{3}H]-phosphoinositol (panel c) fractions is shown. For further experimental details see Materials and Methods. Values shown are the means ± SD of triplicate determinations from one representative experiment of three similar experiments.

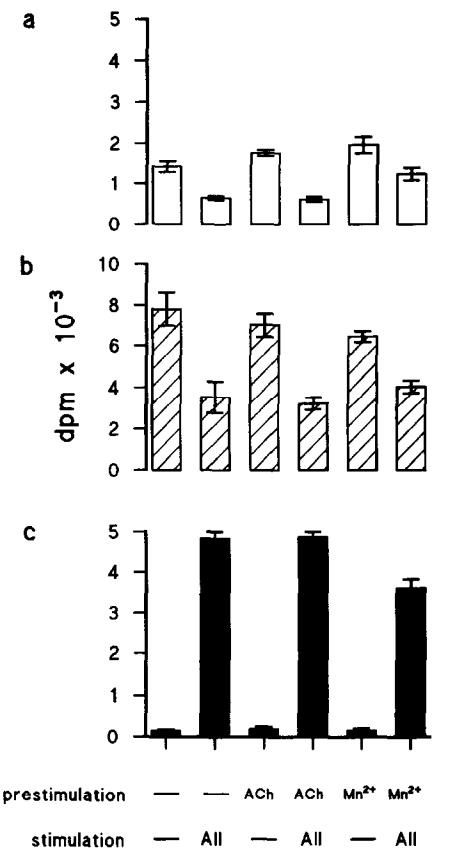

Fig. 2. Effect of ACh and Mn^{2+} pre-stimulation on subsequent chronic AII-stimulated redistribution of radioactivity between [^{3}H]phosphoinositide, [^{3}H]inositol and [^{3}H]phosphoinositol fractions of pre-labelled bovine zfr cells. Cells were pre-labelled for 6 hr with [^{3}H]inositol in the presence or absence of Mn^{2+} or ACh, as described. Cells were subsequently incubated for a further 2 hr in the presence or absence of AII (10^{-7} M) in fresh medium containing 10 mM Li^+. The distribution of radioactivity is shown as in Fig. 1. Values shown are the means ± SD of triplicate determinations from one representative experiment of three similar experiments.

achieved by subsequent challenge with AII (10^{-7} M) was not significantly different to that observed in control cells. Thus, the same steady state (see Discussion) was achieved on acute stimulation with AII, whether cells were pre-incubated with ACh or not. In addition, no significant difference in the phosphoinositol response to acute AII stimulation was observed between control and ACh pre-treated cells (c).

Pre-labelling cells with [^{3}H]inositol for 6 hr in the presence of Mn^{2+} resulted in an increased incorporation of label into the phosphoinositides (a) accompanied by a correspondingly reduced free inositol lablelling (b) (both $P < 0.05$). However, on subsequent acute (15 min) stimulation with AII (10^{-7} M), the incremental increases in the labelling of the phosphoinositides (a) and phosphoinositols (c), and the loss of [^{3}H]inositol (b) were not significantly different from those measured in cells labelled in the absence of Mn^{2+}.

Figure 2 shows the changes in the distribution of radioactivity between the three [^{3}H]inositol pools in cells chronically stimulated with AII (2 hr) following pre-incubation with ACh or Mn^{2+}, as above. Chronic AII stimulation resulted in a loss of radioactivity from the phosphoinositide and free [^{3}H]inositol pools (a,b), and a correspondingly large phosphoinositol response (c) (all $P < 0.05$). Although pre-labelling in the presence of ACh increased incorporation of [^{3}H]inositol into the phosphoinositides (a) ($P < 0.01$), the level to which phosphoinositide labelling and free [^{3}H]inositol levels dropped in response to subsequent chronic AII stimulation (a) was unaf-

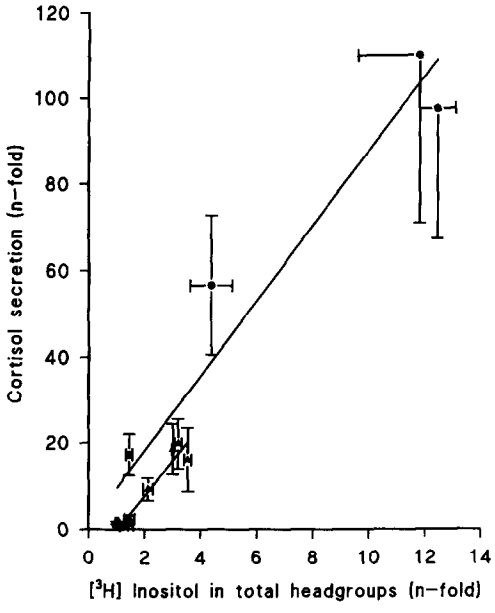

Fig. 3. Correlation between AII- and ACh-stimulated cortisol secretion and phosphoinositol production in primary cultures of bovine zfr cells. Cortisol secretion (over 1 hr) and phosphoinositol production (over 15 min) were measured in response to ACh (10^{-9}–10^{-3} M) (▲) and AII (10^{-11}–10^{-7} M) (●) as described. Results are expressed as *n*-fold stimulation ratios, and show the linear relationship between cortisol secretion and phosphoinositidase C activation (as measured by the accumulation of aqueous-labelled phosphoinositol headgroups) for both agonists. Regression analysis of the data gave correlation coefficients (*r*) of 0.972 and 0.964 for ACh and AII, respectively. The gradients of the regression lines were not significantly different (8.38 ± 1.07 and 8.34 ± 1.2, respectively). Values shown are the means ± SEM of data from 3–6 experiments, each performed in triplicate.

fected by ACh pre-stimulation, as was the magnitude of the phosphoinositol response to AII (c). In contrast, when cells pre-labelled in the presence of Mn^{2+} were subsequently stimulated chronically (2 hr) with AII in the presence of Li^+, the elevated level of phosphoinositide labelling in Mn^{2+} pre-treated cells (a) was decreased on AII stimulation by the same amount, but not down to the same level, as cells not pre-treated with Mn^{2+}. In addition, the magnitude of the phosphoinositol response to AII was reduced following Mn^{2+} pre-stimulation compared with that seen in both control and ACh pre-treated cells ($P < 0.01$).

We have demonstrated previously that both ACh and AII produce dose-dependent increases in cortisol secretion in bovine zfr cells, accompanied by a corresponding activation of phosphoinositidase C with similar dose dependency [6]. Figure 3 illustrates the correlation between phosphoinositidase C activation (as measured by the accumulation of water-soluble [^{3}H]phosphoinositol headgroups over 15 min, in cells pre-labelled to steady state) and the cortisol response (over 60 min) for both AII (10^{-11}–10^{-7} M) and ACh (10^{-9}–10^{-3} M). A strong correlation was revealed between these two responses for each agonist (correlation coeffecients: ACh, $r = 0.972$ and AII, $r = 0.964$; both $P < 0.01$). Moreover, the gradients of the regression lines for AII and ACh were not significantly different (8.32 ± 1.21 and 8.34 ± 1.07, respectively).

DISCUSSION

The effects of AII and ACh on phosphoinositide resynthesis and subsequent breakdown were examined in cells pre-labelled with [^{3}H]inositol for 6 hr. We have previously shown that cells labelled for this length of time contain large reserves of [^{3}H]inositol in the cytosol but only incorporate small amounts of label into the phosphoinositides [13]. Because cells are not labelled to steady state, resynthesis of phosphoinositides (as a consequence of agonist-stimulated phosphoinositide turnover) increases labelling of the phosphoinositide pool. If stimulation is also carried out in the presence of Li^+ to prevent recycling of the phosphoinositols into inositol, AII (10^{-7} M) stimulates incorporation to a new steady state within 15 min. This new steady state is maintained for up to 30 min of stimulation. After 30 min, cytosolic [^{3}H]inositol becomes depleted so that newly synthesized lipid is catabolized without resynthesis. Thus, at stimulation times longer than 30 min, breakdown of this newly labelled lipid can be observed [13].

Figure 1 shows the effect of ACh and Mn^{2+} pre-stimulation on the subsequent redistribution of [^{3}H]inositol in response to acute AII stimulation. Although ACh preincubation increased phosphoinositide labelling, the maximal incorporation of label into the phosphoinositide pool in response to AII was not significantly different between control and ACh pre-treated cells. This is consistent with the stimulation by ACh of incorporation of [^{3}H]-inositol into the same hormone-sensitive phosphoinositide pool as that utilized by AII (i.e. if ACh had stimulated labelling of a different pool than that acted upon by AII, then the label incorporation into phosphoinositides would be greater with ACh preincubation than without). Consistent with this conclusion, the magnitude of the phosphoinositol response to acute AII stimulation in the presence of Li^{2+} was unaffected by ACh pre-stimulation during labelling.

We have also studied the effects of pre-labelling cells in the presence of 1 mM Mn^{2+}. Treatment with Mn^{2+} has been shown in other cells to stimulate labelling by both phosphatidylinositol synthase activity and inositol headgroup exchange [15–17]. However, this process appears to stimulate the labelling of a hormone-insensitive pool of inositol phospholipid [18]. Pre-labelling zfr cells with [^{3}H]-inositol in the presence of Mn^{2+} increased the incorporation of label into the phosphoinositides and reduced free inositol labelling. However, unlike ACh pre-stimulation, the subsequent acute AII stimulation resulted in a significant increase in the maximum phosphoinositide labelling, though the incremental increase in phosphoinositide and phosphoinositol labelling, and the loss of [^{3}H]inositol were not significantly different from those measured

in control cells (labelled in the absence of Mn^{2+}). Thus, these findings are consistent with Mn^{2+}, unlike ACh, promoting the labelling of a phosphoinositide pool distinct from that acted on by AII.

ACh pre-stimulation had no effect on the level to which phosphoinositide labelling and free [^{3}H]-inositol dropped in response to subsequent chronic AII stimulation, and did not alter the magnitude of the accompanying phosphinositol response (Fig. 2). Thus, the lipid synthesized in response to the ACh pre-stimulation was accessible to the phosphoinositidase C activated by AII stimulation and therefore must have been in the same hormone sensitive pool. In contrast, when cells pre-labelled in the presence of Mn^{2+} were subsequently stimulated chronically with AII in the presence of Li^+, the pool of phosphoinositide labelled in response to Mn^{2+} during the pre-incubation remained inaccessible to the AII-stimulated phosphoinositidase C. Cells pre-treated with Mn^{2+} showed the same incremental decrease in phosphoinositide labelling in response to chronic AII stimulation as did control cells. These findings also support stimulation by Mn^{2+} of the labelling of a hormone-insensitive pool of phosphoinositide.

It could be argued that the hormone-insensitive pool of [^{3}H]inositol phospholipid labelled in the presence of Mn^{2+} represents labelling of 3-phosphorylated phospholipids. However, HPLC analysis of the phosphoinositols formed in response to AII stimulation in bovine zfr cells indicates that 3-phosphate derivatives of the phosphoinositides are not formed [19]. Even if 3-phosphorylated phosphoinositides are not substrates for phosphoinositidase activity [20], FPLC and HPLC studies of the deacylated phosphoinositides from bovine zfr cells have also failed to detect multiple isomeric forms of phosphatidylinositol bisphosphate (I.M.B., unpublished data).

We have shown previously that ACh and AII activate a hormone-sensitive phosphoinositidase C, and stimulate the mobilization of a common intracellular pool of calcium. The data presented here reveal that both ACh and AII also lead to the breakdown and resynthesis of a common hormone-sensitive pool of inositol phospholipid. A similar situation appears to occur in rat zona glomerulosa cells for vasopressin and AII [21], and in WRK-1 cells stimulated with bradykinin and vasopressin [22].

We also demonstrate (Fig. 3) that the gradients of the cortisol versus [^{3}H]inositol headgroup responses for ACh and AII were not significantly different in cells labelled to steady state. Such a finding is consistent with both ACh and AII acting through a common mechanism and common pool of phosphoinositide to promote steroidogenesis. The coupling of two or more receptors to a common pool of phosphoinositide in the same cell has implications with respect to the possible modulation of the effects of one agonist by the other. Homologous sensitization or desensitization to either agonist must occur through changes at the level of the receptor, whereas heterologous desensitization would be expected to result from changes in the phosphoinositidase C–G protein complex.

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