J. steroid Biochem. Vol. 27, No. 4–6, pp. 737–743, 1987
Printed in Great Britain.

0022-4731/87 $3.00 + 0.00

Proceedings of the VII International Congress on Hormonal Steroids (Madrid, Spain, 1986)

AN UPDATE ON THE ROLE OF PHOSPHOLIPID METABOLISM IN THE ACTION OF STEROIDOGENIC AGENTS

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Summary—Most steroidogenic agents which bind to cell surface receptors activate adenylate cyclase and/or phospholipase C. Activation of either signaling system may also be associated with rapid increases in *de novo* phosopholipid synthesis, but it is at present uncertain whether this is a secondary or parallel event. Activation of phospholipase C leads to hydrolysis of phosphatidylinositol-4′,5′-PO_4 (PIP_2) and generation of two second messengers, inositol-triphosphate and diacylglycerol (DAG), which mobilize Ca^{2+} and activate protein kinase C, respectively. Increases in *de novo* phospholipid synthesis lead to rapid increases in phosphatidic acid, DAG and C-kinase activity. The PIP_2-phospholipase C system appears to initiate the steroidogenic response to certain agents, such as angiotensin-II, and this may be amplified by concomitant increases in phospholipid synthesis. With other agonists, the role of phospholipase C activation and *de novo* phospholipid (and DAG) synthesis is less certain. In some tissues, activation of protein kinase C by exogeneously added DAG analogues provokes an increase in steroidogenesis. However, this is not observed in other tissues, and it is uncertain whether this rules out involvement of the C-kinase system for steroidogenesis in these tissues, or whether endogenously produced DAG is a more effective activator of the relevant C-kinase system then exogenously added DAG analogues. The role of other potential intracellular signaling substances that may be derived from phospholipase C activation and *de novo* phospholipid synthesis is also at present uncertain, as are the interrelationships between these two phospholipid responses, cyclic nucleotides, and other steroidogenic factors.

Virtually every steroidogenic agent provokes rapid and at times dramatic changes in phospholipid metabolism, but the relationship of these changes to steroidogenesis and other cellular processes remains enigmatic. Those agents, which activate cell surface receptors and primarily use Ca^{2+} rather than adenosine-3′,5′-monophosphate (cAMP) to stimulate steroidogenesis, apparently activate a phospholipase C (Fig. 1), which hydrolyzes phosphatidylinositol-4′,5′,-(PO_4) (PIP_2) and increases: (a) inositol-trisphosphate (IP_3), causing Ca^{2+} mobilization from internal stores; and (b) diacylglycerol (DAG), causing activation of protein kinase C (PKC). In addition, this phospholipase C activation may, in some systems, be associated with large increases in *de novo* phospholipid synthesis from glycerol-3′-PO_4 and fatty acyl-coenzyme A (Fig. 1). Those agents which activate cell surface receptors and primarily activate adenylate cyclase and use cAMP to stimulate steroidogenesis, may also concomitantly increase *de novo* phospholipid synthesis. We have recently found the latter to be attended by increases in DAG and PKC activity, in the absence of increases in IP_3 and consequent Ca^{2+} mobilization. In this case, DAG is derived directly from phosphatidic acid (PA) by PA phosphatase action.

Angiotensin-II (A-II) is an excellent example of a steroidogenic agent which primarily activates the PIP_2–phospholipase C system [1–4]. In dispersed adrenal glomerulosa cells from rat and bovine sources, there is clear-cut activation of this system and no apparent increases in cAMP [5]. ACTH in the rat adrenal, on the other hand, is an excellent example of a steroidogenic agent which primarily increases cAMP [6], and concomitantly increases *de novo* phospholipid synthesis [7–10], DAG and PKC activity (unpublished observations). Curiously, we have recently found evidence for mild, transient ACTH-induced activation of the PIP_2 phospholipase C system in the rat [11], but not bovine adrenal (unpublished) and this is apparent only at lower ACTH concentrations. It is of interest that increases in cyclic-GMP have also been reported to occur transiently at lower ACTH concentrations [12, 13], and it is possible that phospholipase C-induced activation of PKC and/or Ca^{2+} may activate guanylate cyclase [14]. Conceivably, at higher ACTH concentrations, increases in DAG by the *de novo* effect, or large increases in cAMP (particularly in static adrenal cell incubation systems), may serve to turn off the phospholipase C activation, perhaps through effects on GTP-regulatory proteins. If this is true, it would appear that at least some steroidogenic hormones have the potential to act via either (or both) of these two intracellular signaling systems, and other modulatory factors may serve to determine which system will predominate.

Dual activation of the PIP_2–phospholipase C and adenylate cyclase systems seems to occur much more frequently than had been anticipated. For example, activation of the PIP_2–phospholipase C system has recently been shown to occur in the actions of two other agents which before now had only been thought to activate adenylate cyclase, viz. secretin in

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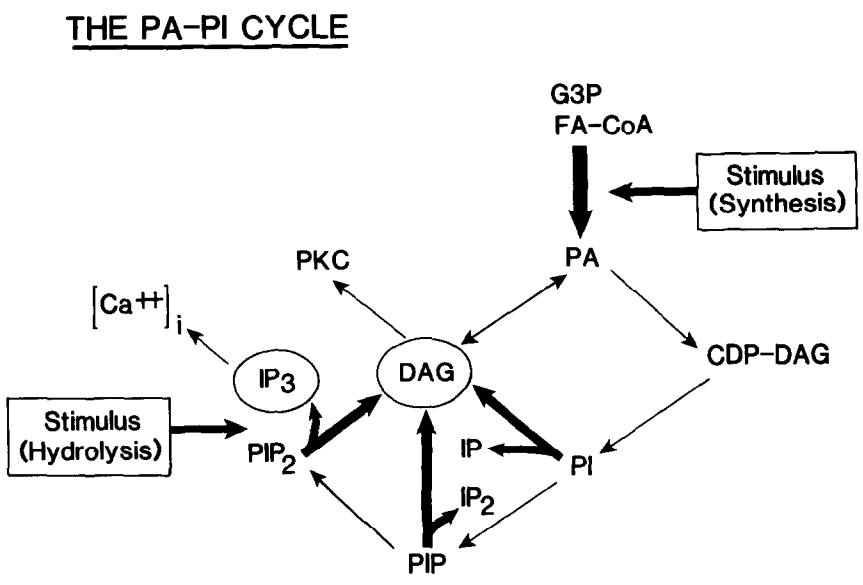

Fig. 1. The phosphatidate–phosphatidylinositol cycle: G3P—glycerol-3'-PO_4, Fa-CoA—fatty acyl-coenzyme A; PA—phosphatidic acid; CDP-DAG—cytidine diphosphate-diacylglycerol; PI—phosphatidylinositol; PIP—phosphatidylinositol-4'-PO_4; PIP_2—phosphatidylinositol-4',5'-$(PO_4)_2$; IP—inositol-phosphate; IP_2—inositol-bisphosphate; IP_3—inositol-trisphosphate; DAG—diacylglycerol; PKC—protein kinase C.

the pancreatic acinar cells [15], and luteinizing hormone (LH) in boving corpus luteal [16] and rat ovarian granulosa [17] cells. In the case of LH in ovarian cells, activation of the PIP_2–phospholipase C system is (unlike ACTH in the adrenal) not transient and follows a dose dependency on LH, which is indistinguishable from that of cAMP. This activation of PIP_2–phospholipase C is not due to LH-induced increases in cAMP. Dual activation of PIP_2–phospholipase C and cAMP systems by LH, on the other hand, may not be apparent in other target tissues, or cell preparations thereof. For example, in rat Leydig cells, activation of PIP_2–phospholipase C was not so apparent, as there were no LH-induced increases in $^{32}PO_4$ incorporation into PA and phosphatidylinositol (PI), as would be expected from hydrolysis of PIP_2 and resynthesis of PA and PI via DAG. In these cells, the only apparent phospholipid effect of LH appeared to be an increase in *de novo* phospholipid synthesis, as evidenced by increased contents of PA, PI, PIP and PIP_2 [18]. These results suggested that LH in rat Leydig cells primarily employs the cAMP system, with an associated increase in *de novo* phospholipid synthesis. Whether these differences in activation of the PIP_2–phospholipase C and cAMP systems reflects differences in receptors or their coupling factors is at present unknown.

The stimulation of *de novo* phospholipid synthesis does not necessarily imply prior or concomitant increases in cAMP. This may indeed be the case in the actions of ACTH in the rat adrenal [7–10] and LH in the rat Leydig cell [18], where cAMP increases, and either accompanies or causes [7, 8, 18] the apparent increases in phospholipid content. Such increases in PA, PI, PIP and PIP_2 content may in fact be due to both an initial burst of synthetic activity, as was observed with cAMP treatment of adrenal sections [19], coupled with a subsequent decrease in degradative turnover of these lipids, possibly via inhibition of phospholipase C [20]. The initial burst of synthetic activity may be due to a rapid increase in glycogenolysis and glycolysis, thus supplying glycerol-3-PO_4 as substrate for *de novo* PA synthesis. Increases in *de novo* phospholipid synthesis also occur in the actions of insulin and AII, which do not appear to increase cAMP. With AII and insulin, increases in glycolysis also occur, albeit by differing mechanisms, and perhaps this glycolytic flux is important for increasing *de novo* phospholipid synthesis. [Note—the insulin effect does not depend on extracellular glucose [21], and is either due to enhanced glycolysis or increased glycerol-3-PO_4 acyltransferase activity, or both.] In keeping with this possibility, glucose itself has been shown to directly and rapidly increase *de novo* phospholipid (PA, DAG, PI, PIP) synthesis in pancreatic islets [22, 23], which are freely permeable to glucose. We have also observed in some systems that when intracellular glycolytic flux is increased, synthesis and contents of PA, PI, PIP and PIP_2 concomitantly increase, and the *de novo* effect on these substances may not longer be apparent in agonist action, presumably because this cycle is already saturated (unpublished observations). However, we have found that the newly synthesized PA may be shunted directly to DAG, away from PI, PIP and PIP_2 in these circumstances (Fig. 1). Obviously, this DAG may activate PKC.

It is not all that surprising that *de novo* phos-

pholipid synthesis is commonly activated in conjunction with activation of phospholipase C. In pancreatic acinar tissue, it is known that acetylcholine increases both hydrolysis [24] and *de novo* synthesis of PA and PI [25], with hydrolysis apparently predominating (since PI levels decrease). Both effects have also been observed with stimulation of insulin secretion in pancreatic islets [22, 23, 26]. Presumably, in these cases, the *de novo* synthesis effect serves to insure that PI–PIP–PIP_2 reserves are replenished sufficiently to provide for continued generation of IP_3 and DAG, and thus maintain the exocytotic secretory response. In addition, the *de novo* effect may directly increase DAG via PA and thus activate PKC.

For steroidogenic agents, the importance of PIP_2 hydrolysis by phospholipase C, and consequent increases in IP_3, Ca^{2+}, DAG and PKC activity, seems most apparent in the stimulation of aldosterone secretion in adrenal cells by AII. Combined actions of Ca^{2+} ionophores, such as A23187, and PKC activators, such as phorbol esters or other DAG analogues [3, 4], result in apparently full mimickry of AII effects on aldosterone secretion. Furthermore, if the PA–PI cycle is interrupted by using Li^+, which inhibits the phosphatase that cleaves inositol–PO_4, and diminishes the availability of free inositol for PI resynthesis, AII effects on aldosterone are diminished [27]. The latter result also suggests that the *de novo* phospholipid synthesis effect of AII [1, 28, 29], where apparent, may contribute importantly for aldosterone synthesis by replenishing or expanding PI, PIP and PIP_2 reserves, thus intensifying increases in IP_3, Ca^{2+} and DAG.

Although AII provokes a dramatic increase in PI, PIP and PIP_2 contents in intact rat adrenal capsular tissue *in vivo* [1] and *in vitro* [28, 29], this *de novo* response may no longer be evident when these cells are dispersed (see Refs. [2, 30] and unpublished observations). The reason for apparent loss of the *de novo* response is unknown, but could be due to loss of specific receptors or coupling factors, cell–cell communication factors, or artefactual increases in glycogenolysis or glucose uptake and glycolytic flux (thus increasing synthesis of PA–PI–PIP–PIP_2 in the controls). It is at present uncertain whether the *de novo* response is fully lost, or diminished, and occurs only to an extent which allows for replenishing PI–PIP–PIP_2 reserves that have been depleted by phospholipase C action [2]. In any event, it seems clear that absolute increases in PI, PIP, and PIP_2 contents are not an obligatory requirement for the increases in steriodogenesis that are apparent in these dispersed cells. However, the true importance of the *de novo* effect in AII-induced steroidogenesis is unknown, as it remains distinctly possible that the *de novo* effect contributes importantly, particularly in intact adrenal tissues.

The importance of *de novo* phospholipid synthesis in ACTH (or LH) action in the rat adrenal (or Leydig cell) also remains uncertain. We and others [31], have documented that increases in the contents of PA, PI and PIP (and other phospholipids) occur in response to ACTH and cAMP, and these increases seem to be well correlated with increases in steroidogenesis, and, like the latter, are inhibited by cycloheximide. We have shown that polyphosphorylated phospholipids can increase cholesterol side-chain cleavage when added to mitochondria *in vitro* [32, 33]. As shown in Table 1, this stimulation is best observed when PIP is added along with free cholesterol, or when PIP is added to cycloheximide-treated mitochondria, which are rich in free cholesterol. (This may explain some failures to see increases in pregnenolone synthesis when PIP is used alone.) Similar stimulation of cholesterol side-chain cleavage was found upon adding PIP to a purified cytochrome $P450_{scc}$–phospholipid vesicle system [34]. Unfortunately, the relevance of these *in vitro* findings is questionable, particularly in view of the high PIP concentrations required to elicit these effects and the finding [31] that PIP is probably not found in adrenal mitochondria. Thus, if ACTH increases steroidogenesis by phospholipid-depen-

Table 1. Effects of phosphatidylinositol-4′-PO_4, cholesterol and phosphatidylcholine on pregnenolone synthesis in rat adrenal mitochondria

Incubation components	ng Pregnenolone/mg protein
Control mitochondria	1.01 ± 0.09 (5)
Control mitochondria, +200 μM PIP	1.43 ± 0.13 (6)
Control mitochondria, +200 μM PIP + 100 μM cholesterol	3.41 ± 0.80 (8)
Control mitochondria, +200 μM PC	0.94 ± 0.10 (8)
Control mitochondria, +200 μM PC + 100 μM cholesterol	1.10 ± 0.09 (8)
ACTH + CH mitochondria	1.36 ± 0.14 (4)
ACTH + CH mitochondria, +200 μM PIP	2.62 ± 0.27 (6)
ACTH + CH mitochondria, +200 μM PIP + 100 μM cholesterol	4.16 ± 0.61 (6)

PIP—Phosphatidylinositol-4′-PO_4; PC—phosphatidylcholine; CH—cycloheximide. Adrenal mitochondria from control or ACTH + cycloheximide-treated rats were prepared and incubated for 10 min at 37°C as described previously [32, 33] with or without PIP, PC and cholesterol, as indicated. Pregnenolone synthesis was measured as described previously [32, 33]. Mean ± SE. The number of determinations is shown in parentheses.

dent effects, other potential mediators may be involved, e.g. (a) increases in other polyphosporylated phospholipids such as phosphatidylglycerol–PO_4, which is present in mitochondria, (b) activation of the DAG–PKC system, or (c) generation of other undefined mediators (see below).

With respect to activation of the DAG–PKC system, this would be expected to occur at lower ACTH doses [11] with mild, transient activation of the phospholipase C which hydrolyzes PIP_2. However, higher doses of ACTH also appear to activate the DAG–PKC system. For example, we have found that 15 min after IP injection of 2 U of ACTH *in vivo*, rat adrenal DAG is increased 2–3-fold; in addition, there is a decrease in cytosolic PKC activity, and stoichiometric increase in membrane-bound PKC activity (submitted for publication). Our findings suggest that, in the rat adrenal gland, ACTH, via DAG, activates PKC and promotes its translocation to membranes. Based upon the failure of higher concentrations of ACTH to increase inositol, mono-, di- and triphosphates (Fig. 2), the increase in DAG observed at higher ACTH concentrations may be derived largely from *de novo* phospholipid synthesis, rather than phospholipase C-mediated hydrolysis of PIP_2, PIP and PI. Whatever the source of the DAG, the importance of PKC activation in steroidogenesis, unfortunately, remains uncertain, since activation of PKC by phorbol esters does not increase steroidogenesis in rat adrenal cells (Fig. 3). Whether or not this negative finding rules out a role for PKC in steroidogenesis is, however,

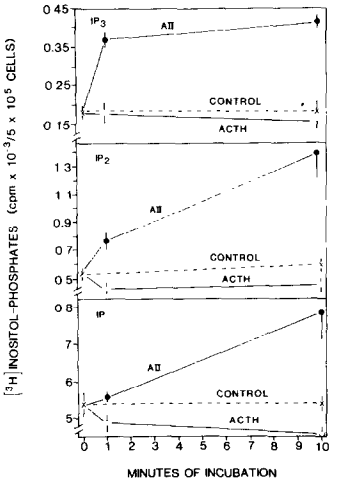

Fig. 2. Failure of high concentrations of ACTH (10^{-6} M) to increase inositol-phosphate production in rat adrenal fasciculata-reticularis cells. Cells were prelabeled with [^{3}H]inositol for 90 min, washed and incubated alone (control) or with 10^{-6} M ACTH or 10^{-4} M angiotensin-II (AII) for the indicated times. [Note—AII was used as a positive control in these experiments; we believe that this effect of high concentrations of AII occurs in fasciculata-reticularis cells, as there was minimal contamination by glomerulosa (<5%) or medullary (<10%) cells.] Mean ± SE of 4 determinations.

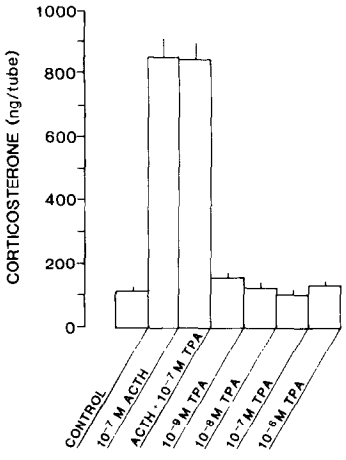

Fig. 3. Failure of 12-O-tetradecanoyl-phorbol-13-acetate (TPA) to alter steroidogenesis in rat adrenal-fasciculata cells incubated with or without ACTH. Mean values ± SE of 4 determinations.

also uncertain, as there are multiple forms of PKC [35], and phorbol ester-induced PKC activation may not truly mimic that of ACTH. To add further confusion to the picture, phorbol esters have been found to stimulate steroidogenesis in bovine [36] and Y-1 [37] cells. ACTH also alters PKC activity or distribution in bovine and Y-1 cells, but we have failed to observe some of the more obvious effects of ACTH on either *de novo* phospholipid synthesis or PIP_2–phospholipase C activation in these cells (unpublished observations). It remains to be determined whether ACTH provokes more subtle changes in phospholipid metabolism in these cells, or whether the observed alterations in PKC system are due to non-phospholipid-dependent factors.

The possibility that there may be other, more subtle phospholipid changes occurring (and other mediators operating) during ACTH action, is suggested by a recent finding that the *de novo* phospholipid synthesis effect of insulin in BC3H-1 myocytes is associated with a concomitant activation of a specific phospholipase C that hydrolyzes a PI-containing glycolipid, yielding a water-soluble phosphoinositol–carbohydrate derivative, and a myristate-rich DAG species [38, 39]. The former substance has been postulated to function as a "second messenger" and directly activate certain enzyme systems, whereas DAG may activate PKC and indirectly alter other cellular processes during insulin action.

Of further interest, we have recently found that the *de novo* phospholipid synthesis effect of insulin is decidedly more complex than we have originally envisioned (unpublished observations). Over the first minute of insulin treatment in BC3H-1 myocytes, there are rapid, large increases in [^{3}H]glycerol incorporation into PA, DAG, PI, and other lipids and phospholipids. After 1 min, increases in [^{3}H]

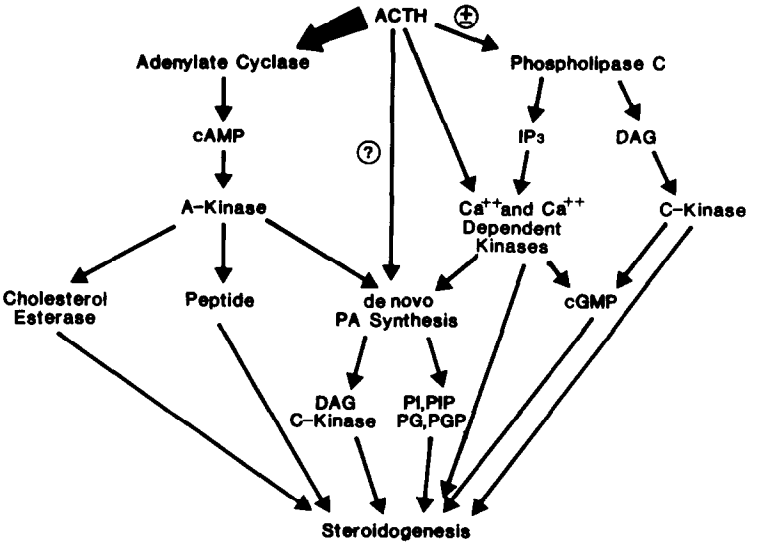

Fig. 4. Multifactorial control of steroidogenesis.

radioactivity in DAG and PA persist, whereas [^{3}H]PI diminishes. PI levels (i.e. mass) remain elevated, and, and may be derived, via DAG and PA, from phospholipase C acting upon other unlabeled phospholipids. After 10–20 min, [^{3}H]glycerol incorporation into PI and other phospholipids increases again. Our results suggest that insulin provokes a rapid 1-min burst of *de novo* phospholipid synthesis, followed by a second period (1–20 min) of lipid remodeling, and by a third period of *de novo* phospholipid synthesis. During all periods, DAG content (as per mass and ^{3}H labeling) is maintained, apparently both by *de novo* phospholipid synthesis and phospholipase C-mediated hydrolyses of PIP_2 (only fleetingly) or other phospholipids, including the PI-glycan. We have yet to examine these possibilities during the action of ACTH and other steroidogenic agents that increase *de novo* phospholipid synthesis.

One fact that emerges from the above considerations is the likelihood that steroidogenesis, particularly in more intact, non-tumorous tissues, where there are no losses of receptors, coupling factors and consequent mechanisms, is a multifactorial process (Fig. 4). The contribution of each mechanism to the steroidogenic action of each agonist in each of its target tissues remains to be determined. Furthermore, the interrelationships between these mechanisms must also be elucidated. For example, does cAMP inhibit the PIP_2–phospholipase C system? Does cAMP stimulate the DAG–PKC system, either by phospholipid or non-phospholipid effects? Do ACTH and other steroidogenic agents increase *de novo* phospholipid synthesis via cAMP or Ca^{2+}, or by a more direct mechanism at the plasma membrane? Is increased glycolytic flux responsible for increases *de novo* phospholipid synthesis? Does *de novo* phospholipid synthesis increase steroidogenesis in some systems by increasing the availability of inositol–phospholipids which serve as substrates for phospholipase C-mediated generation of intracellular signaling substances, IP_3 and DAG? Does *de novo* phospholipid synthesis stimulate steroidogenesis by supplying DAG directly through PA? Is cycloheximide sensitivity of steroidogenesis due to the fact that steroidogenic peptides are labile and must be newly synthesized, or is this sensitivity due to activation of a non-specific phospholipase C [40] that results in inhibition of certain enzymes or depletion of phospholipid reserves that are required to generate signals for steroidogensis? (Interestingly, the latter explanation seems to fit with the finding that insulin effects on pyruvate dehydrogenase are rapidly lost with cycloheximide treatment [21], a situation suspiciously analogous to cycloheximide-induced reversal of steroidogenesis. Conceivably, cycloheximide may deplete that PI which is necessary to synthesize the PI-glycan and thus its derivative, the phosphoinositol-glycan [38, 39], which may mediate insulin's effect on pyruvate dehydrogenase.) Does activation of the cAMP or PIP_2–phospholipase C system lead to an increase in a common steroidogenic peptide, or are there different mechanisms for increasing steroidogensis? Are there other phospholipases and other intracellular mediators involved in the steroidogenesic response?

From the above discussion, it is clear that while much has been learned in the last few years, our understanding of the mechanisms which control steroidogenesis is still very limited. However, on a more positive note, we are at least more aware of some of the remaining questions.

Acknowledgements—This work was supported by funds from the Research Service of the Veterans Administration and the American Heart Association.

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