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ras-Transformation of MDCK Cells Alters Responses to Phorbol Ester Without Altering Responses to Bradykinin

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The results of studies to evaluate the hypothesis that the 21 kDa GTP-binding protein derived from the *ras* oncogene is involved in regulation and coupling of hormone receptors to phospholipase activity have thus far been inconsistent. We therefore examined the effect of H-*ras* transformation on basal, tumor-promoting phorbol ester (TPA)-stimulated, and bradykinin-mediated phospholipid hydrolysis in Madin Darby canine kidney cells (MDCK) by comparing H-*ras*-transformed MDCK cells (MDCK-RAS) to two non-transformed strains of MDCK cells (MDCK-D1 and MDCK-ATCC). In unstimulated MDCK-RAS, diacylglycerol (DAG), inositol phosphate accumulation, and choline phosphate release were increased while arachidonic acid and arachidonic acid metabolite (AA) release was not increased, suggesting that *ras* transformation increased phospholipase C activity. Protein kinase C (PK-C) activity was decreased, and specific binding of [^{3}H]phorbol ester was reduced in MDCK-RAS relative to the non-transformed MDCK cells suggesting that elevated DAG may activate and thereby down-regulate PK-C. Consistent with this finding in MDCK-RAS, TPA-stimulated AA release and subsequent prostaglandin E_2 production were decreased, while TPA-stimulated choline phosphate release was increased. Bradykinin receptor-stimulated phospholipid hydrolysis in MDCK-RAS was similar to that of non-transformed cells, suggesting that the *ras*-derived protein does not directly couple bradykinin receptors to phospholipases in MDCK cells. However, the ability of TPA-treatment to inhibit bradykinin-stimulated phosphoinositide hydrolysis and enhance bradykinin-stimulated AA release was attenuated in MDCK-RAS. Additionally, in MDCK-RAS the

Abbreviations used: AA, arachidonic acid and arachidonic acid metabolites; BK, bradykinin; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminotetraacetic acid; EGTA, (ethylenebis (oxyetheleneonytrilo) tetraacetic acid; HEPES, 4-2-hydroxyethyl-1-piperazineethanesulfonate; IP, inositol phosphates; MDCK, Madin Darby canine kidney; NP40, Nonidet P40; PC, phosphatidylcholine; PDBu, phorbol dibutyrate; PGE_2, prostaglandin E_2; PI, phosphoinositides; PK-C, calcium/phospholipid-dependent protein kinase, protein kinase C; TPA, 12-o-tetradecanolylphorbol-13-myristate acetate.

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conversion of arachidonic acid to prostaglandin E_2 was substantially reduced. We conclude that *ras* transformation of MDCK cells increases DAG levels, thereby activating and, in turn, down-regulating PK-C and certain responses to TPA. Since activation of PK-C may result in a variety of effects on signal transduction pathways, we propose that increased DAG and altered PK-C levels associated with *ras* transformation may account for the inconsistent effects previously observed in studies evaluating the effect of *ras* transformation on phospholipases and other signal transduction systems.

Key words: phorbol esters, *ras*, diacylglycerol, phospholipid hydrolysis, arachidonic acid

Presence of the *ras* oncogene has been correlated with the transformation and the oncogenic potential of certain cells and tumors [for review, see 1]. The 21 kDa protein produced by the *ras* protooncogene is located in the plasma membrane, hydrolyzes GTP, and thus resembles the signal-transducing guanine nucleotide regulatory (G) protein family [for review, see 2]. G proteins couple cell surface receptors to effector enzymes such as adenylyl cyclase and phospholipase C. By analogy, a role for *ras*-derived protein has been sought in signal transduction. In yeast, *ras*-related proteins stimulate adenylyl cyclase [3]. However, in mammalian cells, *ras* transformation does not appear to be associated with altered adenylyl cyclase activity [4,5] but instead has been correlated with both increased [6–9] and decreased [9,10] activity of phospholipase C as well as altered activity of phospholipase A_2 [11,12]. Therefore, studies that have sought to ascribe a role for the *ras* protein in regulation of phospholipid hydrolysis have been inconsistent.

The current studies were designed to resolve some of these inconsistencies. We, thus, evaluated the effect of transformation with H-*ras* on phospholipid hydrolysis in MDCK cells. MDCK were derived from the distal tubule/collecting duct of a normal dog kidney and are considered to be a well-differentiated cell type that retains transport capabilities characteristic of kidney cells [13]. A clonal isolate, MDCK-D1, of the heterogenous MDCK-ATCC has provided a useful system for the elucidation of the mechanisms by which $alpha_1$-adrenergic and bradykinin receptors mediate the activations of phospholipase C and phospholipase A_2 [14–16]. In MDCK cells, the phorbol ester, TPA, activates protein kinase C, and this activation can regulate both the activity of phospholipase A_2 and subsequent PGE_2 production [17]. Additionally, in MDCK cells [18] as well as other cell types [19], TPA enhances the hydrolysis of phosphatidylcholine by the activation of phospholipase C and/or D.

MDCK cells have been transformed by the Harvey murine sarcoma virus (MDCK-RAS) [4] and have been shown to produce high levels of the transforming gene product, H-*ras* [20]. MDCK-RAS exhibit altered responses to glucagon and vasopressin, normal cAMP generation in response to beta adrenergic agonists and prostaglandins [21], and decreased production of PGE_2 [20]. In the current studies, we examined the effect of *ras* transformation on basal, bradykinin-mediated, and TPA-mediated phospholipid hydrolysis and protein kinase C by comparing MDCK-RAS to the two well-characterized non-*ras*-transformed strains of MDCK (MDCK-ATCC and MDCK-D1). Using this approach, we have developed a hypothesis to explain the inconsistent effects of *ras* transformation on phospholipid hydrolysis in other cell types.

MATERIALS AND METHODS

MDCK-ATCC were obtained from the American Type Culture Collection. H-*ras* transformed MDCK cells (clone T-10) were obtained from Dr. M. Lin (National

Institutes of Health) and are designated as MDCK-RAS in this report. Increased levels of the $p21^{ras}$ gene product was previously shown [20] and confirmed by immunoblotting in our laboratory.[1] MDCK-D1 were clonally derived by limiting dilution of MDCK-ATCC [22]. The cells were maintained in DMEM supplemented with 2.5% fetal calf serum and 7.5% horse serum and 15 mM HEPES. For experiments cells were grown to 70–90% confluence in 35 mm dishes (3–4 days). Under these conditions all cell lines were actively growing and not contact inhibited. Unless otherwise indicated, experiments were performed with triplicate 35 mm dishes.

Methods for assay of AA release, PI hydrolysis, PGE_2 production, and analysis of the components of [^{3}H]AA release by thin-layer chromatography have been reported previously [14,16]. The method for the measurement of phosphatidylcholine hydrolysis and quantitation of [^{3}H] DAG levels have been reported in detail [15]. Thin-layer chromatography to identify the relative amounts of [^{3}H]choline and [^{3}H]choline phosphate produced was performed as previously described [23]. The basal and stimulated (i.e., TPA or bradykinin) data were normalized by factoring the tritiated product (i.e., inositols, choline metabolites, or arachidonic acid metabolites) to the tritium incorporated by the cells. Labeling with the tritiated precursor in these assays was carryed out with DMEM containing 0.5% serum for 24 hr unless otherwise indicated. Typical unstimulated (basal) cpm are shown in Table IV. For the measurement of tritiated DAG, MDCK cells were preincubated in DMEM containing either 0.5% serum to be comparable to these assays or 10% serum, to estimated DAG levels in actively growing cells.

PK-C activity was assayed in the three cell types by determining ^{32}P incorporation into histone III-S (lysine-rich histone fraction). The cell monolayers (100 mm dishes) were washed twice with ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 2 mM DTT), scraped into 2 ml of lysis buffer containing 1% NP-40, and homogenized with 20 strokes of a tight-fitting Dounce homogenizer. The homogenates were centrifuged at 40,000g for 60 min at 4°C. The supernatants were applied to DEAE-Sephacel columns (1.5 ml bed volume) previously equilibrated with column buffer (20 mM Tris HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 2 mM DTT). After washing with column buffer (10 ml) PK-C activity was eluted with column buffer containing 0.15 M NaCl (5 ml). PK-C activity in the eluate was determined in a reaction mixture containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.6 mM EGTA, 0.6 mM EDTA, 30 mM NaCl 60 ng/ml phosphatidylserine, 6 ng/ml diolein, 5.6 mM DTT, 1 mg/ml histone III-S, and 50 μM ^{32}P-ATP (approx. 1,000 cpm/pmol). The phosphorylation reaction proceeded for 5 min at 30°C and was stopped by adding the reaction mixture to phosphocellulose P-81 paper (Whatman) as previously described [24]. All determinations were performed in duplicate, and PK-C activity was defined as ^{32}P incorporated in the presence of phosphatidylserine and diolein and expressed as pmol ^{32}P incorporated/min/10^6 cells, or pmol ^{32}P incorporated/min/μg eluted protein. Protein concentration in the column eluates was determined by the method of Lowry et al. [25].

[^{3}H]PDBu binding to intact cells was measured essentially as previously described [26]. Briefly, cell monolayers were washed twice with DMEM supplemented with 20 mM Hepes and 1 mg/ml bovine serum albumin (incubation medium) and then incubated for 30 min at 37°C with 750 μl incubation medium containing 10–80 nM [^{3}H]PDBu in the presence or absence of 20 μM PDBu. The binding reaction was stopped by aspirating the medium; then the cells were washed twice with 1 ml of ice-cold

incubation medium, solubilized in 500 μl 1 M NaOH, neutralized with 500 μl 1 M HCl, and bound [^{3}H]PDBU was determined by liquid scintillation counting. Under these conditions specific binding of [^{3}H]PDBu reached a plateau after 5 min and remained unchanged for at least 60 min. Specific binding of phorbol dibutyrate was normalized to cell number.

TPA, histone III-S, NP40, AA, and BK were obtained from Sigma. DEAE-Sephacel was from Pharmacia. [^{3}H]2-myo-Inositol (10–20 Ci/mmol) was from American Radiolabeled Chemicals., Inc. [^{3}H]AA acid (10–100 Ci/mmol), [methyl-^{3}H]choline chloride (60–90 Ci/mmol), [^{3}H]PDBu (12–19) Ci/mmol, ^{32}P-ATP (3,000 Ci/mmol), and PGE_2 radioimmunoassay kit were from DuPont-New England Nuclear. DMEM was from GIBCO. Scintillation counting was conducted in a Beckman scintillation counter with Liquisint (National Diagnostics) to yield a 40% efficiency of tritium counting. All other materials were from standard sources.

RESULTS

Increased DAG and Altered Levels of PKC

Increased DAG levels have been observed in *ras*-transformed cells [6,8,27], and it has been suggested that this increase results from increased phospholipase C-mediated hydrolysis of phospholipids, such as the PI [6], PC, or phosphatidylethanolamine [28,30]. We found that MDCK-RAS cells incubated with [^{3}H]AA had DAG levels (expressed as a ratio of [^{3}H]DAG to [^{3}H]phospholipid) that were two- to fivefold higher than in MDCK-D1 and MDCK-ATCC; this increase was similar whether cells were preincubated in the presence of 0.5% (Fig. 1A) or 10% (Fig. 1B) serum.

Enhanced formation of DAG in *ras*-transformed cells could result in persistent activation and thereby down-regulation of PK-C. Decreased PK-C activity as measured by histone IIIs phosphorylation was seen in MDCK-RAS relative to the non-transformed cells (Table I). The activity in MDCK-RAS was decreased by more than 40% relative to MDCK-D1 or MDCK-ATCC whether expressed on a cell number or μg

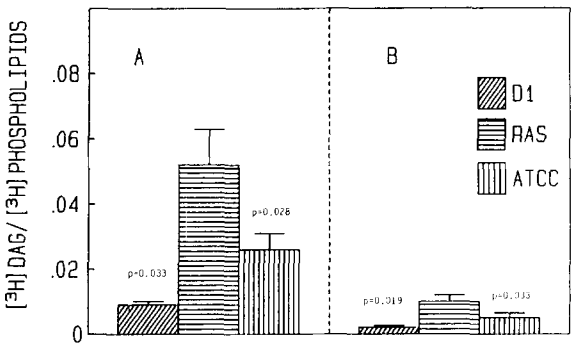

Fig. 1. MDCK-RAS have increased diacylglycerol (DAG) levels. Cells were preincubated for 24 hr in DMEM (0.5% fetal calf serum) with 1 μCi/ml [^{3}H]arachidonic acid (**A**) or for 24 hr in DMEM (10% serum) with 2 μCi/ml [^{3}H]arachidonic acid (**B**). For both A and B cells were washed and extracted with cold MeOH. The extracts were analyzed for [^{3}H]DAG as described in "Materials and Methods." [^{3}H]DAG ranged from 308 to 1,444 cpm in A and from 190 to 3,992 cpm in B. Data shown are expressed as the ratio of [^{3}H]DAG/[^{3}H]phospholipids and represent the mean ± SEM of 3 independent experiments. The *P* values shown above the bars are statistical significance by paired one-tailed t test of the difference between MDCK-RAS and non-transformed cells.

TABLE I. Protein Kinase C Activity*

	D-1	RAS	ATCC
pmole ^{32}P/min/10^6 cells	62.67 ± 6.6	15.9 ± 2.0	44.0 ± 4.6
pmole ^{32}P/min/μg eluted protein	1.02 ± 0.02	0.6 ± 0.12	1.15 ± 0.1

*Protein kinase C activities were measured by histone IIIs phosphorylation as described in "Materials and Methods." The data are expressed as pmole ^{32}P incorporated/min per cell No. or per μg protein eluted from DEAE sephacel columns. Data shown are the mean ± S.E. for three independent experiments assayed in duplicate.

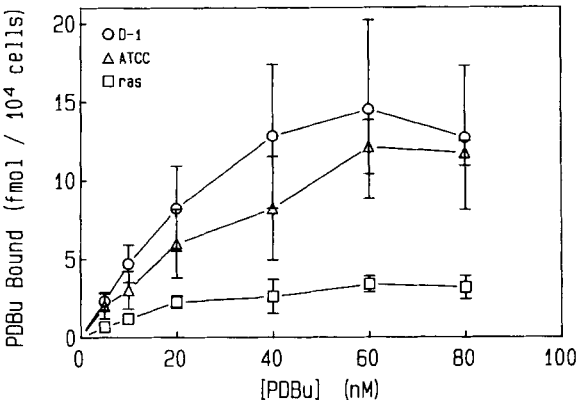

Fig. 2. Decreased phorbol dibutyrate binding in MDCK-RAS. Washed cell monolayers were incubated with [^{3}H]PDBu at the indicated concentrations ± 20 μM PDBu for 30 min at 37°C. Specific binding was determined in the solubilized monolayers. Results shown are mean ± SEM of three independent experiments.

protein basis. In support of the finding that *ras*-transformation decreases PK-C activity, we found that specific binding of the phorbol ester [^{3}H]PDBu was reduced in MDCK-RAS relative to non-transformed cells (Fig. 2). The reduction in binding was approximately equivalent at all concentrations of PDBu used, precluding the possibility that the elevated DAG level per se competitively inhibited the binding. Thus, the elevated DAG levels in MDCK-RAS appear to result in down-regulation of PK-C as has been observed in *ras*-transformed NIH 3T3 cells [8].

In unstimulated MDCK-RAS cells, total IP accumulated intracellularly over 40 min were twice the values of non-*ras*-transformed MDCK cells (Fig. 3A). Similarly, the release of [^{3}H]choline and choline phosphate in a 1 hr period was greater than that of non-transformed cells (Fig. 3B). These results, together with the data showing increased DAG levels, suggest that activity of one or more types of phospholipase C is increased in MDCK-RAS. By contrast, the release of [^{3}H]AA over a 1 hr period in unstimulated MDCK-RAS cells was not increased (Fig. 3C); this result suggests that unlike phospholipase C activity, phospholipase A_2 activity is not enhanced in MDCK cells by *ras* transformation.

TPA-Stimulated Activation of Phospholipid Hydrolysis

A possible consequence of persistent activation of PK-C in *ras*-transformed cells would be altered responses to the phorbol ester TPA [31,32]. Previous studies in MDCK

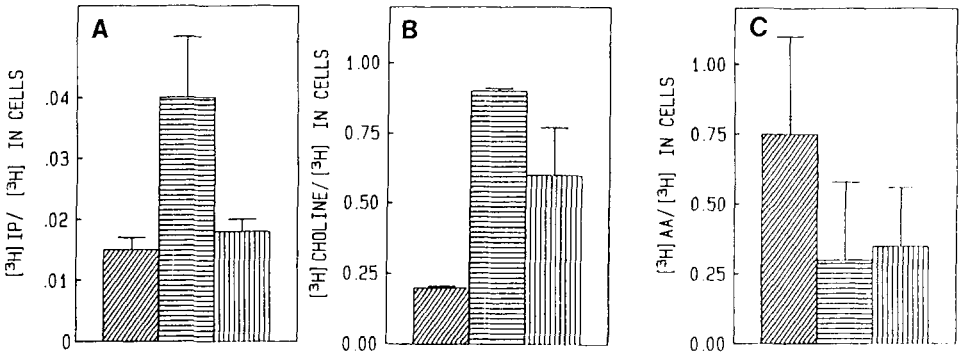

Fig. 3. Increased production of inositol phosphates (IP) and choline metabolites but not AA in MDCK-RAS. **A:** The level of [^{3}H]IP was determined after washed cells were incubated in DMEM with 50 mM LiCl for 40 min. Data shown are the mean ± SE of three independent experiments and are expressed as the total intracellular [^{3}H]IP/total [^{3}H]inositol containing phospholipids. [^{3}H]Inositol incorporated into lipids for MDCK-D1, MDCK-RAS, and MDCK-ATCC was 0.9 ± 0.02, 1.2 ± 0.05, and 1.4 ± 0.15 $\times 10^6$ cpm, respectively. **B:** The level of [^{3}H]choline metabolites released by washed cells incubated in DMEM (0.05% BSA) for 1 hr. Data shown are the mean ± S.E.M. of 3 independent experiments and expressed as total [^{3}H]choline metabolite release/[^{3}H]choline associated with the cells. [^{3}H]Choline associated with the cells for MDCK-D1, MDCK-RAS and MDCK-ATCC was 2.1 ± 0.15, 2.0 ± 0.08, 1.5 ± 0.03 $\times 10^6$ cpm, respectively. **C:** The level of [^{3}H]AA released by washed cells incubated in DMEM (0.05% BSA) was measured over a 1 hr period. Data shown are the mean ± S.E. of 2 independent experiments and expressed as total [^{3}H]AA released/[^{3}H] arachidonic acid associated with the cells. [^{3}H]Arachidonic acid associated with MDCK-D1, MDCK-RAS, and MDCK-ATCC was 1.7 ± 0.02, 1.7 ± 0.2, 1.8 ± 0.15 $\times 10^6$ cpm, respectively. The significance of the difference between MCDK-RAS and non-transformed cells, as determined by paired one-tailed t test, was $P < .05$ for A and B and $P > .05$ for C.

cells have indicated that TPA, acting via PK-C, regulates the activity of phospholipase A_2 and the conversion of arachidonic acid to PGE_2 [17]. Release of [^{3}H]AA (expressed as a percent of cell-associated label released by a 60 min TPA treatment) was markedly reduced in MDCK-RAS (Fig. 4, left panel). This reduction was not a result of altered kinetics or an altered concentration dependence, since these were similar for both *ras*-transformed and non-transformed MDCK cells (data not shown). In both MDCK-D1 and MDCK-ATCC, TPA-stimulated [^{3}H]AA release consisted of less than 25% free arachidonic acid with the remainder being arachidonic acid metabolites (Table IIA). However, in MDCK-RAS, TPA-stimulated [^{3}H]AA release consisted primarily of free arachidonic acid; in support of this observation, MDCK-RAS produce negligible amounts of PGE_2 (as assayed by radioimmunoassay) in response to TPA (Table IIB). These data suggest that regulation of phospholipase A_2 and the conversion of arachidonic acid to PGE_2 by PK-C is altered in MDCK-RAS.

In contrast to TPA-stimulated [^{3}H]AA release, TPA-stimulated release of [^{3}H]choline and choline phosphate (expressed as a percent of total cell associated [^{3}H]choline) was increased in *ras*-transformed cells (Fig. 4, right panel). This increase was not a result of altered kinetics or an altered concentration dependence (data not shown). [^{3}H]Choline phosphate was the primary metabolite released from TPA-stimulated cells (Table III), suggesting that TPA activates phospholipase C (and not phospholipase D) and that this activation occurs similarly in *ras*-transformed and non-transformed cells.

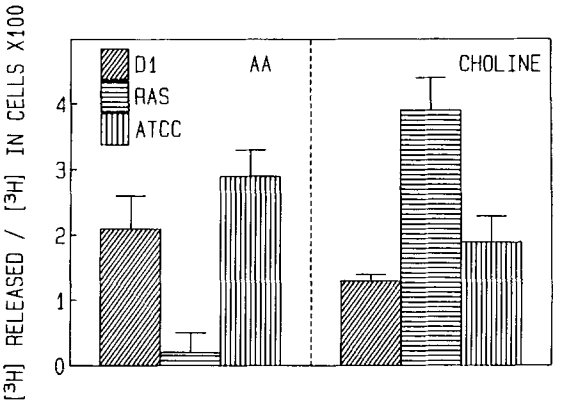

Fig. 4. TPA-stimulated [^{3}H]AA release and [^{3}H]choline release in MDCK-D1, MDCK-ATCC, and MDCK-RAS cells. Cells were incubated with 0.33 μCi/ml [^{3}H]arachidonic acid for 4 hr (**left**) or 5 μCi/ml [^{3}H]choline (**right**) for 24 hr. Washed cells were incubated with TPA (100 nM) for 1 hr and 30 min for [^{3}H]AA and [^{3}H]choline release, respectively. Data are expressed as a ratio of total [^{3}H]AA released to tritium associated with the cells and represent the mean ± S.E.M. of 3 independent experiments. Typical values for incorporation of radiolabeled precursors are shown in Figure 3.

TABLE II. The Effect of *ras* Transformation on [^{3}H]Arachidonic Acid and [^{3}H]Arachidonic Acid Metabolite Release and Prostaglandin E_2 Formation in MDCK Cells (MDCK-D1, MDCK-RAS, MDCK-ATCC)*

A.

		cpm			% total cpm		
Cells	Stimulus	AA	PGE_2	Other	AA	PGE_2	Other
D1	BK	613	1,883	699	19	59	22
RAS	BK	7,692	1,109	1,431	75	11	14
ATCC	BK	3,132	296	799	7	74	19
D1	TPA	7,431	11,962	4,609	17	57	26
RAS	TPA	1,983	220	202	82	9	8
ATCC	TPA	1,589	11,627	4,207	9	67	24

B.

			PGE_2 pg/dish		
Stimulant		Time	D1	RAS	ATCC
Arachidonic acid	10 μM	30′	13,860	4,560	15,000
	1 μM	30′	11,860	Not detectable	11,000
	0.1 μM	30′	1,060	Not detectable	7,000
Bradykinin (1 μM)		10′	263	61	2,353
TPA (100 nm)		2 hr	428	12	1,300

*A. Cells were incubated with 0.33 μCi/ml [^{3}H]arachidonic acid for 4 hr, washed, and incubated with bradykinin (1 μM) for 15 min or TPA (100 nM) for 90 min. The components of [^{3}H]AA were analyzed as described in "Materials and Methods." Data shown are the mean cpm (stimulated-basal) of triplicate dishes from a representative experiment that was replicated twice.

B. Cells were washed and stimulated for the times indicated below. PGE_2 production into the medium was assayed by radioimmunoassay. The pg/dish formed are shown from a representative experiment that was replicated twice.

TABLE III. Components of [^{3}H]Choline/Choline Phosphate Release in MDCK Cells (MDCK-D1, MDCK-RAS, MDCK-ATCC)*

Cells	Stimuli	[^{3}H]choline phosphate	[^{3}H]choline
D1	BK	1,030	Not detectable
RAS	BK	15,960	2,660
ATCC	BK	10,900	5,050
D1	TPA	35,920	780
RAS	TPA	92,850	4,370
ATCC	TPA	80,960	2,020

*Cells were incubated with 5 μCi/ml [^{3}H]choline for 24 hr, washed, and then incubated with bradykinin (1 μM) or TPA (100 nM) for 60 min. The cpm of choline and choline phosphates were determined as described in "Materials and Methods." Data shown are the results obtained in a representative experiment that was replicated twice.

TABLE IV. The Effect of *ras* Transformation on Bradykinin-Stimulated Responses in MDCK Cells (MDCK-D1, MDCK-RAS, MDCK-ATCC) *

A.

Response	Percent basal control			(Range of basal) cpm × 10^3
	D-1	RAS	ATCC	
[^{3}H]AA	1,242 ± 142 (n = 3)	920 ± 113 (n = 3)	636 ± 85 (n = 3)	0.2–1
[^{3}H]IP	160 ± 6 (n = 7)	140 ± 7 (n = 6)	150 ± 11 (n = 4)	5–10
[^{3}H]choline	156 ± 1 (n = 3)	239 ± 7 (n = 3)	138 ± 9 (n = 3)	8–20

B.

	$\frac{\text{BK response (+TPA)}}{\text{BK response (−TPA)}} \times 100$		
	D1	RAS	ATCC
[^{3}H]AA	158 ± 10 (n = 4)	89 ± 7 (n = 4)	134 ± 3 (n = 4)
[^{3}H]IP	13 ± 4 (n = 6)	79 ± 8 (n = 7)	15 ± 10 (n = 3)

*A. Bradykinin-stimulated (1 μM) [^{3}H]IP formation (40 min), [^{3}H]choline metabolites (20 min), and [^{3}H]AA release (20 min) were assayed as described in "Materials and Methods." Data shown are expressed as the mean ± SE of the percent basal for n = 3–7 independent experiments.
B. Effect of TPA treatment on bradykinin-stimulated [^{3}H]IP formation and [^{3}H]AA release. Cells were treated with TPA (100 nM) for 10 min. Bradykinin-stimulated [^{3}H]IP formation and [^{3}H]AA release were then assayed as in A. Data shown are expressed as the ratio of the response elicited by bradykinin in the presence of TPA over the response elicited by bradykinin in the absence of TPA (×100).

Bradykinin-Stimulated Phospholipid Hydrolysis

It has been postulated that the *ras* protooncogene may couple receptors to phospholipase C or A_2 and that the presence of mutated *ras*, which hydrolyses GTP more slowly than normal *ras*, may alter these responses [1]. To test this hypothesis, bradykinin-stimulated phospholipid hydrolysis was compared in MDCK-RAS and

non-transformed MDCK cells. Bradykinin-stimulated [^{3}H]AA release and total [^{3}H]inositol phosphate formation in MDCK-RAS were similar to values observed in non-transformed cells, while bradykinin-mediated phosphatidylcholine hydrolysis in MDCK-RAS was modestly increased (Table IVA). The release of labeled product from [^{3}H]choline-labeled cells in response to bradykinin was complete by 30 min (data not shown) and was identified primarily as choline phosphate (Table III), suggesting that bradykinin stimulates phosphatidylcholine hydrolysis via the activation of phospholipase C in each cell type. These data suggest that *ras* transformation does not substantially alter the coupling of bradykinin receptors to phospholipases and that the *ras* protein is not likely to couple bradykinin receptors to phospholipases in MDCK cells.

In MDCK-D1 cells, bradykinin-stimulated [^{3}H]AA release represents primarily PGE_2 and other arachidonic acid metabolites [16], but in MDCK-RAS basal PGE_2 production is markedly reduced [20]. Consistent with these observations, bradykinin-stimulated [^{3}H]AA release in MDCK-RAS represented a much greater contribution of free arachidonic acid compared to MDCK-D1 and MDCK-ATCC (Table IIA), and the amount of bradykinin-stimulated PGE_2 formed was also reduced in MDCK-RAS (Table IIB).

In order to test if reduced PGE_2 production represented a decrease in the ability of MDCK-RAS to convert free arachidonic acid to metabolites, cells were incubated with various concentrations of arachidonic acid (Table IIB). The conversion of arachidonic acid to PGE_2 was reduced in MDCK-RAS as compared to non-transformed MDCK cells. Thus, in MDCK-RAS, the activation of phospholipase A_2 by bradykinin appears to be unaltered, while the conversion of free arachidonic acid to PGE_2 is reduced.

In both MDCK-D1 [16] and Swiss 3T3 cells [33] TPA-treatment blocks bradykinin-stimulated inositol phosphate formation while it potentiates bradykinin-stimulated AA release. In the current studies, TPA treatment elicited similar effects in MDCK-ATCC and neither inhibited bradykinin-mediated inositol phosphate formation nor potentiated bradykinin-mediated AA release in MDCK-RAS (Table IVB). Increased concentrations of TPA up to 1 μM and increasing TPA treatment for up to 30 min failed to elicit any changes in bradykinin-stimulated responses in MDCK-RAS (data not shown). From these findings we conclude that protein kinase C-mediated effects on bradykinin-stimulated phospholipid hydrolysis are decreased in MDCK-RAS.

DISCUSSION

The *ras*-derived proteins bear a striking resemblance to members of the G protein family, and thus it has been postulated that these oncogene products function in the signal transduction of hormones that are coupled to phospholipases. Several studies have shown that *ras* transformation results in altered activity of phospholipase C [6–10,12] or phospholipase A_2 [11,12]. In contrast with these reports, we found that in MDCK-RAS, as compared to non-transformed MDCK cells, bradykinin-stimulated phosphoinositide hydrolysis and [^{3}H]AA release were not reduced while bradykinin-stimulated phosphatidylcholine hydrolysis was only modestly enhanced. These data suggest that the *ras* gene product is not the G protein involved in coupling of bradykinin receptors to phospholipases in MDCK cells but do not preclude the coupling of other hormone or growth factor receptors to phospholipases in these cells. In other cell types bradykinin-stimulated phosphoinositide hydrolysis is increased by *ras* transformation; however, this may be due to an increased number of bradykinin receptors [34].

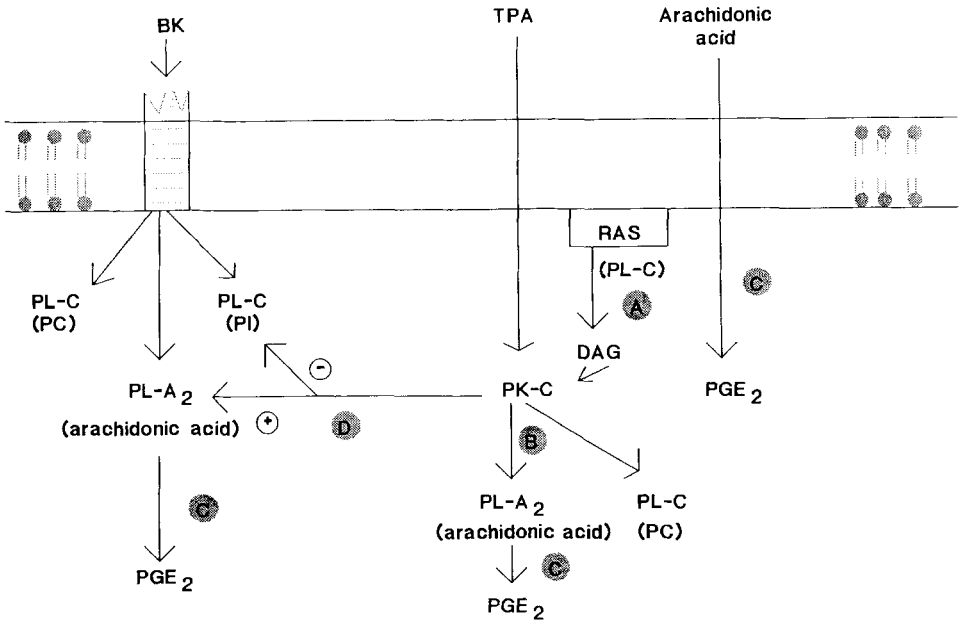

Fig. 5. Proposed mechanism for *ras*-mediated alterations in MDCK cells. **A:** *Ras*-transformation increases phospholipase C (PL-C) activity resulting in increased diacylglycerol (DAG). Increased DAG results in activation of protein kinase C (PK-C). Persistent activation of PK-C results in **B)** decreased PK-C-stimulated phospholipase A_2 (PL-A_2) activation, **C)** decreased conversion of arachidonic acid to PGE_2, and **D)** loss of PK-C mediated effects on bradykinin-mediated phospholipid hydrolysis.

In contrast to bradykinin-mediated stimulation of phospholipases, effects of TPA are strikingly altered in MDCK-RAS (Fig. 5). These alterations include 1) decrease in TPA-stimulated AA release and PGE_2 formation (Fig. 5B,C); 2) lack of TPA-effects on bradykinin-stimulated activation of phospholipases (Fig. 5D); and 3) increased TPA-stimulated phosphatidylcholine hydrolysis. We propose that at least certain (and perhaps all) of these altered responses to TPA in MDCK-RAS are a result of increased DAG and thereby a persistent activation and down-regulation of PK-C.

Nishizuka [32] has suggested that persistent activation of PK-C can result in its down-regulation and that different isoforms may down-regulate at different rates and to different extents. Consistent with this hypothesis are the findings that in MDCK-RAS PK-C activity is decreased, phorbol ester binding is reduced, and some responses to TPA are attenuated while TPA-stimulated phosphatidylcholine hydrolysis is increased.

TPA-stimulated arachidonic acid release is reduced in MDCK-RAS, whereas bradykinin-stimulated arachidonic acid release is not. The latter response appears to result largely from a PK-C-independent activation of phospholipase A_2 [39]. In MDCK-RAS, exogenous arachidonic acid as well as arachidonic acid produced in response to bradykinin or TPA is not as effectively converted to PGE_2 as in MDCK-D1 or MDCK-ATCC cells. It is attractive to speculate that a protein involved in this conversion is altered in response to *ras* transformation and that this protein may contribute to the potentiation of bradykinin-stimulated [^{3}H]AA release by TPA [16,33]. Down-regulation of an 80 kDa protein, which is a PK-C substrate, has previously been observed in *ras*-transformed cells [32].

One explanation for the increased DAG, inositol phosphates, and choline phosphate release in *ras*-transformed cells is that the *ras*-derived protein directly interacts with and stimulates phospholipase C. In support of this hypothesis are data showing that injection of *ras* into *Xenopus* oocytes rapidly increases phosphoinositide-specific phospholipase C [35] and that exogenous *ras* protein can stimulate phospholipase C in membranes labeled with [^{3}H]inositol [36]. However, in membranes from MDCK-RAS, guanine nucleotide-stimulated phosphoinositide hydrolysis is not substantially increased.[1] Alternative but, we believe, less likely explanations are that DAG is increased secondary to transformation itself [27] or that the changes observed are mediated by decreased levels of DAG kinase [37] and/or altered levels of other kinases involved in the metabolism of phospholipids such as choline kinase [38].

Our data suggest that while PK-C itself is not absent in MDCK-RAS cells, certain responses that involve a PK-C pathway are markedly altered. Since PK-C activation has been implicated in the regulation of hormonal responses by mechanisms that include 1) altered properties of receptors [40], 2) alterations in G proteins [41], and 3) changes in effector enzymes or ion channels [42,43], it is likely that a variety of different responses might be observed in cells in which PK-C has been activated. We suggest that a number of the effects that we observe for H-*ras* transformation on phospholipid hydrolysis in MDCK cells are a consequence of activation of PK-C. Therefore, it is likely that effects secondary to PK-C activation, in turn, as a result of increased DAG levels, may alter one or more components in hormone response pathways involving phospholipases and perhaps other signal transduction systems as well. Such effects complicate attempts to determine the precise role of the *ras*-derived protein in signal transduction and thereby provide an explanation for the quite variable responses observed in cells that have been transformed or transfected by *ras*.

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