0026-895X/92/010163-05$03.00/0

MOLECULAR PHARMACOLOGY, 41:163–167

Carbachol-Induced Decrease in Thyroid Cell Adenylyl Cyclase Activity Is Independent of Calcium and Phosphodiesterase Activation

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Received June 6, 1991; Accepted October 18, 1991

SUMMARY

The mechanism of adenylyl cyclase desensitization by carbachol, an agent that stimulates polyphosphoinositide hydrolysis, was studied in thyroid cells. Incubation of cultured dog thyroid cells with 10 μM carbachol for 2–4 hr reduced the subsequent thyrotropic hormone (TSH) stimulation of adenylyl cyclase activity of membrane preparations by ~40%. This inhibition was reversed by atropine, occurred even in a Ca^{2+}-free medium containing ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, and was not reproduced by the Ca^{2+} ionophore A23187. The carbachol effect was not prevented by simultaneous incubation of cells with either isobutylmethylxanthine, an inhibitor of phosphodiesterase, or H-7, an inhibitor of protein kinase. Pretreatment of cells with pertussis toxin to inactivate the G_i inhibitory protein also failed to affect the carbachol inhibition. Although carbachol did not reduce the basal or the TSH-stimulated cyclase activities when added to membranes directly during the assay, exposure of cells to carbachol for 2–4 hr resulted in long lasting inhibition of TSH-stimulated cyclase activity (for at least 24 hr); recovery was seen by 48 hr after its removal. Carbachol pretreatment had no effect on ^{125}I-TSH binding to membranes but reduced the cyclase stimulation by not only TSH but also cholera toxin, guanosine 5′-*O*-(3-thio)triphosphate, and forskolin; it also significantly reduced the cholera toxin-mediated AD[^{32}P]-ribosylation of G_s in membranes. These data indicate that carbachol-induced inhibition of adenylyl cyclase occurs beyond the level of TSH receptor binding and that G_s is a possible site of its action. Thus, in dog thyroid cells, carbachol, via muscarinic receptors, can reduce the adenylyl cyclase activity by a process that does not involve Ca^{2+} or activation of phosphodiesterase.

Inhibition of cAMP accumulation by muscarinic cholinergic stimulation is well known in a variety of cell types (1–4). Although in some systems (5, 6) receptor-mediated inhibition of AC has been described, in thyroid and many other cells it has been shown that the carbachol-induced decrease in cAMP levels involves activation of a Ca^{2+}/calmodulin-dependent PDE (7–12). For thyroid, this conclusion was based on a decrease in cAMP levels when tissue slices (7, 8) or cells (9) were coincubated with carbachol and TSH and the reversal of this inhibition in the presence of an inhibitor of PDE, such as IBMX. This, like other effects of carbachol in the thyroid, was shown to be Ca^{2+} dependent (4, 13). Ca^{2+} has also been implicated in acetylcholine-induced desensitization of glucose oxidation (14) and TSH-induced desensitization of AC (15).

Using the fluorescent Ca^{2+} indicators quin2 and fura-2 in dog thyroid cells, we and others (16–18) have shown that an early step in carbachol action is an increase in intracellular [Ca^{2+}], via the increased inositol-1,4,5-trisphosphate levels resulting from phosphatidylinositol 4,5-bisphosphate hydrolysis (19, 20). We recently observed that phorbol ester treatment of dog thyroid cells mimicked the effect of TSH in causing AC desensitization (21). In that study, incubation of cells with TSH for 2–18 hr or with the phorbol ester TPA for 6–18 hr reduced the AC activity of membrane preparations in response to subsequent TSH stimulation. Using the protein kinase inhibitor H-7, it was shown that the effect of TPA, but not that of TSH, is mediated by the Ca^{2+}/phospholipid-dependent PKC (21). Because carbachol can potentially activate at least two signal-transduction pathways, one via increased $[Ca^{2+}]_i$ and another involving 1,2-diacylglycerol/PKC, we decided to investigate which mechanism was operative in mediating the carbachol-induced AC desensitization in dog thyroid cells. Although it is

This work was supported by United States Public Health Service Grant DK26088 from the National Institutes of Health.

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ABBREVIATIONS: AC, adenylyl cyclase; TSH, thyrotropic hormone; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methyl xanthine; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; TMB-8, 3,4,5-trimethoxy benzoic acid 8-(diethylamino)octyl ether; H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; G_i, inhibitory GTP-binding regulatory protein; G_s, stimulatory GTP-binding regulatory protein; PT, pertussis toxin; CT, cholera toxin; G protein, GTP-binding regulatory protein.

known that carbachol is a potent activator of phosphatidylinositol 4,5-bisphosphate hydrolysis, its effects on 1,2-diacylglycerol formation and PKC activation *per se* in the thyroid have not yet been demonstrated directly.

Thus, as in our earlier studies (21), we have first incubated dog thyroid cells with carbachol under a variety of conditions, prepared membranes, and measured the AC activity in the absence or presence of TSH or other cyclase stimulators. In some experiments, we also measured ^{125}I-TSH binding to membranes, in order to evaluate the effect of carbachol treatment on TSH receptor binding. It should be noted that a major difference between this study and the previous ones with carbachol (4, 7–9) is that, in this study, we did not examine the effect of carbachol on cAMP accumulation in intact cells in the simultaneous presence of a cyclase stimulator such as TSH.

Experimental Procedures

Primary cultures of dog thyroid cells were prepared as described before (21). Cells were cultured in Coon's modified Ham's F-12 medium supplemented with four 'hormones' (TSH, insulin, transferrin, and hydrocortisone), 0.5% bovine calf serum, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin sulfate). Two or three days before their use, cells were transferred to the same medium, devoid of hormones and serum. All cell incubations were performed in this medium, with various additives, for indicated periods of time, at 37° under 5% CO_2. A crude membrane fraction was prepared, and the AC activity and ^{125}I-TSH binding were determined by methods described before (21). AC activity assays were done in triplicate for 10 min at 30°, by measuring the conversion rate of [α-^{32}P]ATP to cAMP in the absence (basal) or presence of TSH (200 milliunits/ml). This dose of TSH produced a maximal response, which typically was a 3–6-fold increase over the basal activity of 100–200 pmol of cAMP formed/mg of protein/10 min. In some experiments, membrane AC activity was also determined in the presence of CT (100 μg/ml) and NAD^+ (1 μM), GTPγS (10 μM), or forskolin (40 μM). GTP (10 μM) was present in all assays of AC, except when GTPγS was used. The results are the mean ± standard error of four or more experiments and were statistically evaluated by analysis of variance followed by Fisher's least significant difference multiple-comparison test. Differences of $p < 0.05$ were considered significant.

Results

Effects on TSH receptor binding and TSH-stimulated AC activity. Addition of carbachol directly to membranes in the AC assay had no significant effect on the basal or the TSH-stimulated AC activity (data not shown), unlike in human thyroid cells (9). However, preincubation of dog thyroid cells with carbachol for various periods significantly reduced the subsequent TSH-stimulated AC activity of membranes; a 30–40% reduction was seen after 2–4 hr, with even greater reductions at 12–18 hr (Fig. 1). Basal AC activity did not change in 2–4 hr, but it was also reduced by 12–18 hr (Fig. 1). Carbachol treatment, however, had no effect on the binding of ^{125}I-TSH to membranes, at any time studied (Fig. 1). Inhibition of TSH-stimulated AC was seen with carbachol doses of 10–100 μM but not with 1 μM carbachol or 100 μM oxotremorine, an agonist of the M_2 subclass of muscarinic receptors (data not shown), suggesting that M2 receptors are not involved in this muscarinic effect. However, simultaneous incubation of cells with carbachol (10 μM) and the potent muscarinic receptor antagonist atropine (30 μM) prevented the inhibitory effect of carbachol (data not shown); TMB-8 (10 μM) had a similar effect

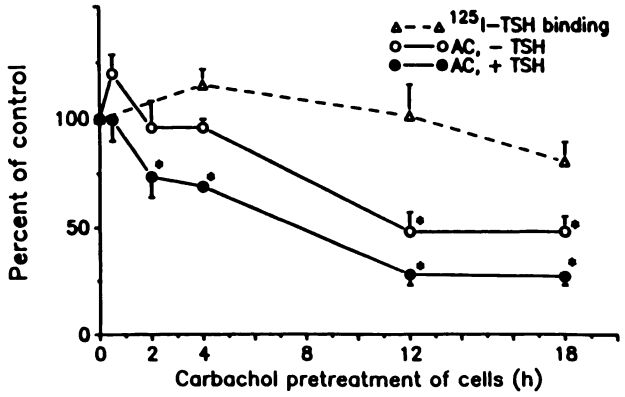

Fig. 1. Kinetics of carbachol effect on AC activity and TSH receptor binding. Cells were treated without or with 10 μM carbachol for the indicated periods, and the membranes prepared were assayed for ^{125}I-TSH binding (Δ) and AC activity in the absence (○) or presence of 10 milliunits of TSH (●). AC activities (pmol of cAMP/mg of protein/10 min) of control membranes were as follows: –TSH, 110 ± 15; +TSH, 680 ± 45 (mean ± standard error, 17 experiments). The specific binding of ^{125}I-TSH (percentage of net ^{125}I-TSH bound/100 μg of protein) to control membranes was 9.7 ± 1.7 (mean ± standard error, 11 experiments). Results (mean ± standard error) are expressed as percentage of corresponding control values; *error bars*, when not seen, are smaller than the *symbol* size. *, $p < 0.05$, compared with control.

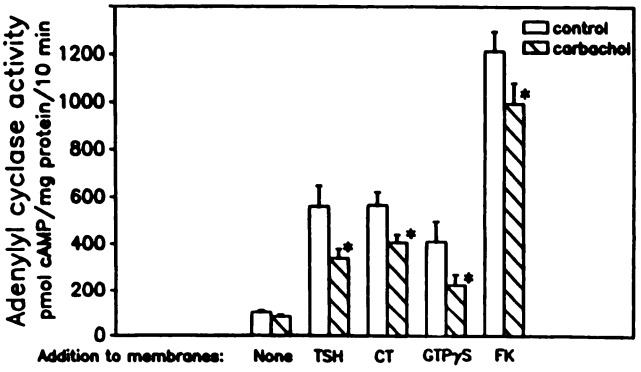

Fig. 2. Effect of carbachol pretreatment on AC stimulation by various agents. Cells were incubated without or with 10 μM carbachol for 4 hr, and the AC activity of membranes was determined in the absence or presence of TSH (10 milliunits), CT (100 μg/ml)/1 μM NAD^+, GTPγS (10 μM), or forskolin (*FK*) (40 μM). Values shown are the mean ± standard error of nine experiments for all treatments (except GTPγS, with four experiments). *, $p < 0.05$, compared with corresponding controls.

as atropine. Although TMB-8 has been used in many systems as an intracellular Ca^{2+} antagonist, recent studies show that this drug may act more as a specific antagonist of muscarinic cholinergic receptors (17), consistent with this study.

Effects on AC stimulation by other agents. Membrane AC activity can be stimulated via different mechanisms by various agents that directly activate G_s or the catalytic unit of AC. Therefore, the effect of pretreatment of cells with carbachol on AC stimulation by such agents was examined. As shown in Fig. 2, carbachol treatment for 4 hr not only reduced TSH stimulation of AC by about 40% but also similarly reduced AC stimulation by CT or GTPγS, both of which directly activate G_s. In addition, carbachol treatment also caused a smaller but significant reduction (by ~18%) in AC response to forskolin, an agent that maximally stimulates AC via direct activation of the catalytic unit (Fig. 2). Interestingly, there was also a significant reduction (by ~25%, $p < 0.05$) in the CT-mediated

ADP-ribosylation of G_s in membranes from carbachol-treated cells, compared with controls (data not shown). In these studies, the [AD[^{32}P]-ribosylation of CT substrates in dog thyroid cell membranes was carried out as described by us before, using [^{32}P]NAD$^+$ (21).

Effects of PDE inhibitor. Because carbachol decreases cAMP levels by activating PDE in many systems, including dog thyroid slices (7, 8), the effect of incubation of cells with carbachol and IBMX, a potent inhibitor of PDE activity, on the TSH-stimulated AC activity of membranes was studied. Carbachol inhibited the TSH-stimulated AC activity to a similar extent both in the absence and in the presence of IBMX (Fig. 3). Although incubation of cells with IBMX alone tended to increase the AC activity of membranes, the result was not statistically significant (Fig. 3).

Ca^{2+} dependence of the carbachol effect. One of the major effects of carbachol in these cells is a rapid elevation of intracellular [Ca^{2+}]; this involves an initial spike of 2–3-fold increase over basal, followed by a sustained increase (for at least 30 min) of ~50% over the resting level of ~170 nM (16–18). Therefore, the Ca^{2+} dependence of the carbachol-induced inhibition of TSH-stimulated AC activity was examined. Cells were incubated in normal Coon's medium (containing 0.9 mM Ca^{2+}), with or without additional EGTA (2 mM), in the absence or presence of carbachol, for 4 hr, and the basal and TSH-stimulated AC activities of membrane preparations were determined. Even in the Ca^{2+}-free medium, carbachol treatment inhibited the TSH-stimulated AC activity similarly as in the presence of Ca^{2+} (Fig. 4). However, we know from earlier studies that the initial spike component of the carbachol-induced rise in $[Ca^{2+}]_i$ still occurs in Ca^{2+}-free medium but can be abolished by pretreatment of cells with 0.5 μM ionomycin (17). Thus, when cells were treated first with ionomycin (0.5 μM) for 5–10 min in Ca^{2+}-free/EGTA medium, followed by carbachol (10 μM) for 4 hr, the inhibitory effect on subsequent TSH-stimulated AC activity was still observed (data not shown). This carbachol effect was not nonspecific, because it was prevented by atropine even in the EGTA-containing medium (data not shown). Furthermore, elevation of $[Ca^{2+}]_i$ by incubation of cells in normal Coon's medium with Ca^{2+} ionophores, 10 μM A23187 (Fig. 4) or 1 μM ionomycin (data not shown), did not reproduce

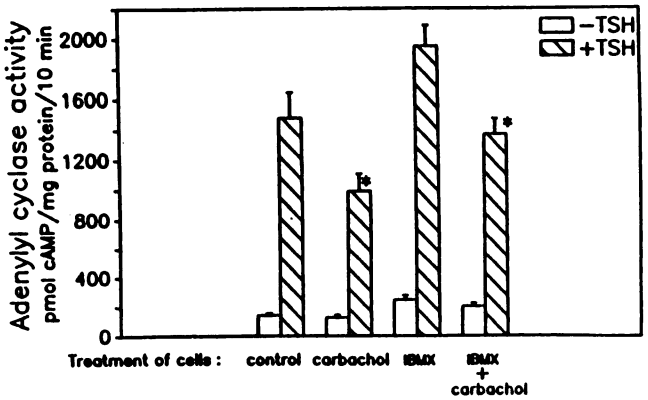

Fig. 3. Effect of PDE inhibitor on carbachol-induced inhibition of TSH-stimulated AC activity. Dog thyroid cells were incubated with 2 mM IBMX for 15 min before addition of 100 μM carbachol for 4 hr. Control cells were incubated for 4 hr without or with carbachol or IBMX alone. The membrane AC activity was determined in the absence (*−TSH*) or presence of TSH (*+TSH*). Values shown are the mean ± standard error of five experiments. *, $p < 0.05$, compared with corresponding control.

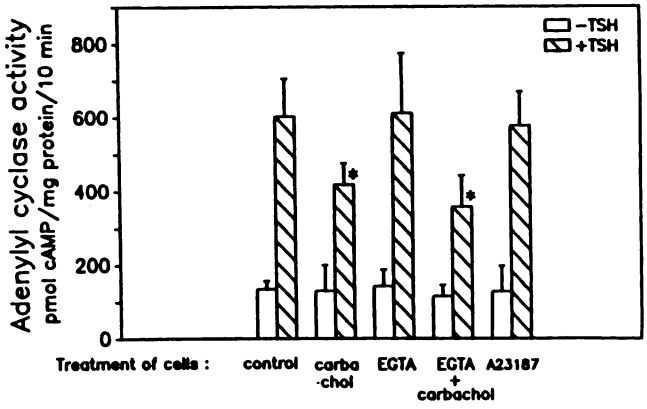

Fig. 4. Lack of dependence on Ca^{2+} of carbachol-induced inhibition of TSH-stimulated AC. Cells were incubated in normal Coon's F-12 medium (containing 0.9 mM Ca^{2+}) or with additional 2 mM EGTA (calculated [Ca^{2+}] = 0.0001 μM), in the absence or presence of 10 μM carbachol for 4 hr. Cells were also incubated in normal medium with a Ca^{2+} ionophore, A23187 (10 μM), for 4 hr. Membrane AC activity was assayed in the absence (*−TSH*) and in the presence of TSH (*+TSH*). Values shown are the mean ± standard error of 7–13 experiments for different treatments. *, $p < 0.05$, compared with corresponding control.

the effect of carbachol. These results suggest that the mechanism of AC inhibition by carbachol pretreatment is independent of Ca^{2+}.

Effect of PKC inhibitor. Our earlier studies on TSH- or TPA-induced desensitization of TSH-stimulated AC showed that the protein kinase inhibitor H-7 reversed desensitization induced by TPA but not by TSH (21). This indicated an involvement of PKC activation in the action of TPA but not that of the hormone. Therefore, we studied the effect of incubation of cells for 4 hr with carbachol in the presence or absence of H-7 (50 μM). Carbachol treatment inhibited TSH-stimulated AC in membranes to a similar extent (~40%) with or without H-7 (data not shown), suggesting that PKC is not involved in this carbachol effect.

Effects of PT pretreatment. Cells were pretreated with PT (1 μg/ml) for 18 hr to inactivate G_i, the inhibitory G protein of the cyclase system; these cells were then incubated with or without carbachol for 4 hr. Carbachol treatment caused a similar reduction in TSH-stimulated AC activity of membranes, with or without PT pretreatment, suggesting that the carbachol effect did not involve G_i activation (data not shown).

Reversibility of the carbachol effect. To study the reversibility of carbachol-induced inhibition of AC activity, cells incubated with 50 μM carbachol for 4 hr were thoroughly washed and reincubated in fresh medium, without carbachol, for 24 or 48 hr; the membrane AC activity was then compared with that of control and 4-hr carbachol-treated cells. Results in Table 1 show that the carbachol effect is long lasting (at least for 24 hr), because cells removed from carbachol for 24 hr still showed a decrease in TSH-stimulated AC activity comparable in extent to that produced by 4-hr carbachol treatment. However, complete recovery from this inhibition occurred by 48 hr (Table 1).

Discussion

The present study has clearly demonstrated that pretreatment of dog thyroid cells with carbachol results in muscarinic receptor-mediated desensitization of AC activity of membranes.

TABLE 1

Recovery from carbachol-induced inhibition of TSH-stimulated AC

Cells were untreated or exposed to 10 μM carbachol for 4 hr and incubated further for 24 or 48 hr. These cells were again either untreated or incubated for 4 hr with 10 μM carbachol before preparation of membranes. AC activity of membranes incubated with or without TSH is expressed as pmol of cAMP formed/mg of protein/10 min. Values are the mean ± standard error of three experiments.

	AC activity			
	−Carbachol		+Carbachol	
	−TSH	+TSH	−TSH	+TSH
	pmol/mg/10 min			
Control	79 ± 20	553 ± 72	61 ± 12	302 ± 26[a]
Exposed to carbachol 24 hr earlier	62 ± 11	371 ± 47[a]	70 ± 13	368 ± 45[a]
Exposed to carbachol 48 hr earlier	72 ± 20	676 ± 93	90 ± 22	452 ± 66[b]

[a] $p < 0.05$, versus +TSH value of untreated control cells.
[b] $p < 0.05$, versus corresponding control.

The AC activity is reduced in response to subsequent stimulation by not only TSH but also other agents, such as CT, GTPγS, and forskolin, which act on different components of the AC system. Further, this carbachol effect is not dependent on an increase in $[Ca^{2+}]_i$, does not involve activation of a PDE, and does not affect TSH receptor binding. Thus, these carbachol effects are different from those of TPA or TSH treatments, which cause TSH receptor down-regulation and AC desensitization to further stimulation by TSH but not CT or forskolin (21). Differences between the effects of carbachol and TSH or TPA are also revealed by the effects of the protein kinase inhibitor H-7, which reversed inhibition by TPA but not that by TSH (21) or carbachol (present study). Although this suggests that PKC is not involved in mediating this effect of carbachol, this observation needs to be confirmed using other doses of and/or longer incubations with H-7 or other PKC inhibitors.

Specific muscarinic receptor-mediated inhibition of TSH-stimulated AC activity by carbachol was indicated by atropine reversal, even in a Ca^{2+}-free medium. Although the carbachol-induced inhibitory effect could be observed in the particulate preparation, it was necessary to treat intact cells with carbachol, because direct incubation of membranes with carbachol produced no inhibition of AC activity in 30 min.

Unlike in other systems (22, 23), G_i activation is not implicated in the carbachol effect, because PT treatment did not eliminate the inhibition of TSH-stimulated AC activity. The possibility that carbachol-induced structural and/or functional alteration of G_s might occur is indicated by the reduction in both CT-mediated AD[^{32}P]-ribosylation of G_s and AC stimulation by CT/GTPγS. However, it should be noted that changes in ADP-ribosylation, suggestive of structural alteration of a G protein, need not strictly correlate with functional changes culminating in AC desensitization. A lack of correlation between changes in AD[^{32}P]-ribosylation and AC desensitization was, indeed, observed in our earlier studies with TPA and TSH (21).

Our results also differ from those of previous studies in dog thyroid slices (4, 7, 8) or human thyroid cells (9), where coincubation with carbachol and TSH resulted in reduced TSH stimulation of cAMP accumulation and that inhibition was abolished either in the absence of extracellular Ca^{2+} or in the presence of an inhibitor of Ca^{2+}/calmodulin-dependent PDE. The reasons for these discrepancies are not presently clear. Indeed, we also found that incubation of dog thyroid slices with carbachol resulted in a similar decrease in TSH-stimulated AC activity of membranes, ruling out possible cell culture artifacts. Our results are more in agreement with those of Champion and Mauchamp (24), who observed a decrease in cAMP levels when pig thyroid cells were coincubated with carbachol and an AC stimulator such as TSH, isoproterenol, or prostaglandin E_2, even in the presence of IBMX.

The exact nature of the mechanism involved in this carbachol action is not known. It is possible that carbachol causes altered membrane function via effects on membrane phospholipid composition. Such involvement of a phospholipid intermediate has been suggested in acetylcholine inhibition of AC in fibroblasts (25). Furthermore, manipulation of membrane phospholipid composition with a nonspecific lipid transfer protein resulted in modification of TSH-stimulated AC activity of bovine thyroid membranes (26), corroborating an earlier proposed role for phospholipids in TSH stimulation of AC (27). Thus, muscarinic regulation of cAMP levels in thyroid appears to be more complex than previously realized; it could occur by mechanisms involving both PDE activation resulting in increased catabolism of cAMP, as proposed earlier (4, 7–9), and an inhibitory process that directly affects AC activity and cAMP synthesis, as shown by the present study. Alternatively, carbachol could also activate phospholipase A_2 and mediate its effects via the metabolites of this pathway; the latter possibility is currently being explored.

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