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1α,25-(OH)$_2$-Vitamin D$_3$ Stimulates the Adenylyl Cyclase Pathway in Muscle Cells by a GTP-Dependent Mechanism Which Presumably Involves Phosphorylation of G$_{\alpha i}$

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To further understand the mechanism underlying 1,25(OH)$_2$D$_3$ activation of the cAMP pathway, the effect of the hormone on adenylyl cyclase (AC), GTPase and protein kinase A (PKA) activities as well as on the phosphorylation of G$_{\alpha i}$ was studied in membranes from chick skeletal muscle cells. The sterol stimulated AC activity in a dose (0.1-10 nM) and time (1-5 min.) dependent fashion, provided GTP (10 μM) was present in the assay. High affinity GTPase activity was unaffected by the hormone. In the absence of GTP or in the presence of Mn^{2+} (20 mM), 1,25(OH)$_2$D$_3$ effects on AC were abolished. PKA activity was increased (+120%) in cells pretreated (1 nM, 5 min.) with the sterol. Moreover, immunoprecipitation of G$_{\alpha i}$ from [^{32}P]-labeled myoblast membranes showed that 5 min. exposure to 1 nM 1,25(OH)$_2$D$_3$ increased (1.5-2 fold) the phosphorylation of its α subunit. The present data suggest that in muscle cells, 1,25(OH)$_2$D$_3$ activates AC by a non direct, GTP-dependent action which could imply amelioration of G$_i$ function by sterol-induced α_i phosphorylation.

The hormonally active form of vitamin D$_3$, 1α,25-dihydroxy-vitamin-D$_3$ (1,25(OH)$_2$D$_3$) affects muscle intracellular calcium levels by two ways: a classic, steroid-like genomic action and a non-genomic (rapid) mechanism which implies direct membrane effects of the hormone (1, 2). Fast actions of 1,25(OH)$_2$D$_3$ involve participation of transmembrane signalling systems resulting in alteration of $^{45}Ca^{2+}$ influx, release of calcium from intracellular stores, cAMP production, modulation of PKC activity and phosphorylation of cellular proteins (2). We have previously shown that 1,25(OH)$_2$D$_3$ rapidly (1-5 min.) stimulates $^{45}Ca^{2+}$ influx in cultured chick embryo skeletal muscle cells (myoblasts) by modulating voltage-dependent Ca^{2+}-channels from the L type (3). This action is completely suppressed by both adenylyl cyclase (AC) and PKA inhibition (4), supporting the concept that the cAMP/PKA pathway mediates rapid hormone effects. Additionally, sterol induced inhibition of a G-protein from the G$_i$ family mediates in part AC stimulation by the hormone (4). This has led to the proposal, as for other cell types, that 1,25(OH)$_2$D$_3$ activation of second messenger systems in muscle cells involves the existence of a putative cell surface receptor for the hormone (1) as proposed for other steroids (5-8). To further characterize the mechanism underlying 1,25(OH)$_2$D$_3$ activation of the cAMP pathway in chick muscle cells, in the present study the effect of the hormone on AC, GTPase and PKA activities as well as on the phosphorylation of G$_i$ was investigated.

MATERIALS AND METHODS

Materials. ATP (GTP-free), GTP, forskolin, lysophosphatidic acid (LPA), isoproterenol and N-ethylmaleimide (NEM) were from Sigma Chemical Co., St.Louis (MO). [^{32}P]H$_3$PO$_4$ was obtained from New England Nuclear, Boston (MA). cAMP radioassay kit was from Diagnostics Products Corp., Los Angeles (CA). All chemicals used were analytical grade.

Cell culture. Chick myoblast cultures were performed as described (3). [^{32}P]-labeling of intact cells and microsomal membrane preparation, as in (4).

Adenylyl cyclase and GTPase activities. AC activity was assayed by incubating the membranes at 30°C in buffer containing 0.5 mM ATP, 10 mM MgCl$_2$ and an ATP-regenerating system, and measuring the in vitro cAMP formation essentially as described (9). High affinity (low K$_m$) GTPase activity was determined as in (10). γ[^{32}P]GTP was synthesized according to (11). Assay medium was identical to that for the AC assay, but 0.1 μM γ[^{32}P]GTP (0.05 μCi/tube) and 0.1 mM unlabeled ATP were included. NEM treatment of membranes was as described in (10).

Protein kinase A activity. PKA activity was measured according to Fujimori et al. (12) and expressed as the PKA activity ratio of the activity measured with or without added cAMP (−cAMP/+cAMP).

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TABLE I

$1,25(OH)_2D_3$-Induced Stimulation of Adenylyl Cyclase Activity

(A) Myoblast membranes were incubated at 30°C for 5 min. in reaction buffer in the presence or absence (basal) of the indicated concentrations of $1,25(OH)_2D_3$, or forskolin (100 μM). AC activity was assayed as described (9). Blank values (boiled membranes) were below 0.1 pmol cAMP/min/mg protein.

	Adenylyl cyclase activity (pmol cAMP/min/mg protein)
Basal	26.3 ± 0.6
$1,25(OH)_2D_3$ (nM)	
0.1	34.4 ± 0.9**
1.0	40.5 ± 0.8**
10.0	33.4 ± 1.8**
Forskolin	72.4 ± 1.0*

(B) Membranes were incubated for the indicated times in reaction buffer in the presence or absence (basal) of $1,25(OH)_2D_3$ (1 nM) or forskolin (100 μM) and AC activity was determined. Data are given as percent of stimulation above basal (100%) AC activity.

	Adenylyl cyclase activity (percent above basal)		
Time	1 min	5 min	10 min
Basal	100	100	100
$1,25(OH)_2D_3$	133 ± 4**	154 ± 3*	162 ± 6*
Forskolin	177 ± 5**	269 ± 7*	381 ± 3*

Data are the average from three independent experiments ± SD. *p<0.001; **p<0.005.

Immunoprecipitation and immunoblotting of $G_{\alpha i}$. Immunoprecipitation and immunoblotting of $G_{\alpha i}$ from [^{32}P]-labelled myoblast microsomal membranes was performed with AS266 antiserum (anti-$G_{\alpha i}$ common, see ref. (13)) a gift from Dr. G. Schultz (Institut fur Pharmakologie, Freie Universitat Berlin, Germany), as described in (14).

Statistical analysis. The statistical significance of data was evaluated using Student's *t*-test (15).

RESULTS

In the presence of $1,25(OH)_2D_3$ in vitro AC activity was increased in a dose (0.1-10 nM)- and time-dependent fashion (Table I), the maximal stimulation being reached at 1 nM (55-60% over basal) after 5 min. of exposure to the sterol. When membranes devoided of endogenous GTP were used (nucleotide free-prepared membranes), the stimulatory action of $1,25(OH)_2D_3$ was strictly dependent on the presence of 10 μM GTP in the reaction mixture (Figure 1), as expected for a G-protein coupled ligand-receptor system. Additionally, AC activity measured in the presence of high concentrations (20 mM) of Mn^{2+} (Table II), a condition which provides information on AC activity in the absence of G-protein regulation (AC catalytic moiety) (16, 17), was unaltered by the hormone. Membranes remained responsive to the diterpene forskolin, although to a lesser extent than in the absence of Mn^{2+}, in accordance with

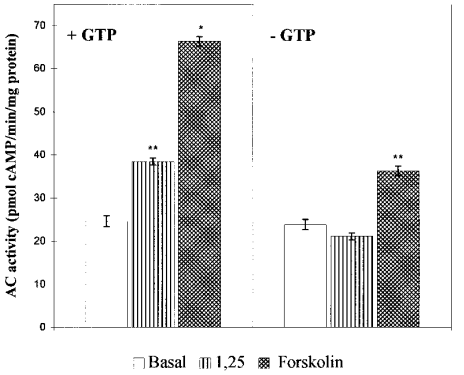

FIG. 1. GTP-dependence of $1,25(OH)_2D_3$-induced stimulation of adenylyl cyclase activity. Myoblast membranes were incubated in reaction buffer with or without 10 μM GTP, in the presence or absence (basal) of $1,25(OH)_2D_3$ ("1,25", 1 nM) or forskolin (100 μM). AC activity was assayed as described in Methods. Data are the average from three independent experiments ± SD. * p<0.001; **p<0.005.

the fact that forskolin ability to activate AC is markedly influenced by G protein function (18). These results rule out a direct action of the sterol on the enzyme. We have previously shown that in muscle cells $1,25(OH)_2D_3$-induced inhibition of G_i is the major mechanism by which the hormone stimulates cAMP production (4). To evaluate if G_s activation by the sterol concomitantly contributes to this action, we measured high affinity GTPase activity in myoblast membranes with or without NEM (100 μM) pretreatment, a condition which improves detection of GTP hydrolysis coming from G_s-coupled agonists (19). As shown (Figure 2), $1,25(OH)_2D_3$ was unable to stimulate high affinity GTPase activity in myoblast membranes, regardless these were treated or not with the alkylating agent. Moreover, 10 μM LPA increased GTPase activity by 39% in control membranes but did not on the NEM-treated preparations, whereas the oppossite occurred for isoproterenol (1 μM) which augmented by 26%

TABLE II

Effect of Mn^{2+} Ion on $1,25(OH)_2D_3$-Induced Stimulation of Adenylyl Cyclase Activity

	Adenylyl cyclase activity (pmol cAMP/min/mg protein)	
	$-Mn^{2+}$	$+Mn^{2+}$
Basal	30.5 ± 0.9	18.7 ± 1.0
$1,25(OH)_2D_3$	48.0 ± 0.9*	17.3 ± 1.8
Forskolin	76.2 ± 1.0*	29.9 ± 2.0**

Myoblast membranes were incubated in reaction buffer with (instead of 10 mM Mg^{2+}) or without 20 mM Mn^{2+} and in the presence or absence (basal) of $1,25(OH)_2D_3$ (1 nM) or forskolin (100 μM). AC activity was determined as described (9).

Data are average from three independent experiments ± SD. *p<0.001; **p<0.005.

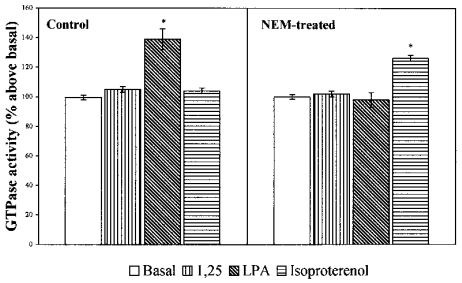

FIG. 2. Effect of $1,25(OH)_2D_3$ on GTPase activity. Control or NEM (100 μM)-treated membranes were incubated in the presence or absence (basal) of $1,25(OH)_2D_3$ ("1,25", 1 nM), LPA (10 μM) or isoproterenol (1 μM). High affinity (low K_m) GTPase activity was determined as described in Methods. Basal, unstimulated GTPase activities were 0.56 and 0.22 pmol of GTP hydrolyzed/min/mg of protein for control and NEM-treated membranes, respectively. Data are the average from three independent experiments ± SD. *$p<0.005$.

GTPase activity in NEM-treated membranes. To evaluate if sterol-dependent AC activity and cAMP generation were translated into PKA activation, the kinase phosphorylating activity was studied. As expected, in cells treated with $1,25(OH)_2D_3$ (1 nM, 5 min.) PKA activity was markedly increased above basal levels (1.06 ± 0.19 vs. 0.48 ± 0.03 PKA activity ratio {-cAMP/+cAMP} for treated vs. control cells, respectively).

In several cell systems a decrease in G_i function correlates with increases in the phosphorylation state of its α subunit (20, 21). To determine if $1,25(OH)_2D_3$ alters the phosphorylation of $G_{\alpha i}$, we employed an anti-$G_{\alpha i}$ antibody (AS266) to selectively immunoprecipitate $G_{\alpha i}$ from [^{32}P]-labeled myoblast membranes. As shown (Figure 3, panel A), pretreatment of cells with the hormone stimulates by 1.5-2 fold the phosphorylation of a 40 kDa membrane protein, which is specifically precipitated by AS266. This antibody selectively recognized a 40 kDa $G_{\alpha i}$ on immunoblotted myoblast membrane preparations (Figure 3, panel B).

DISCUSSION

In previous studies (3, 4, 22) we produced evidence suggesting that cAMP production and PKA activation mediate rapid actions of $1,25(OH)_2D_3$ in skeletal muscle cells. In the present work we further investigated the mechanism underlying sterol induced activation of the AC/cAMP pathway employing membrane preparations from cultured chick skeletal muscle cells. Our data provide the first consistent evidence indicating that in muscle cells, $1,25(OH)_2D_3$ activates AC by a non-direct (receptor mediated?) GTP-dependent (G-protein transduced) action. This is in line with previous work indirectly suggesting G-protein mediated-AC activation by the sterol (4). To our knowledge, this is the first report where the effect of $1,25(OH)_2D_3$ on low K_m (high affinity, G-protein dependent) GTPase activity is studied. GTPase stimulation by G_s-coupled agonists is generally

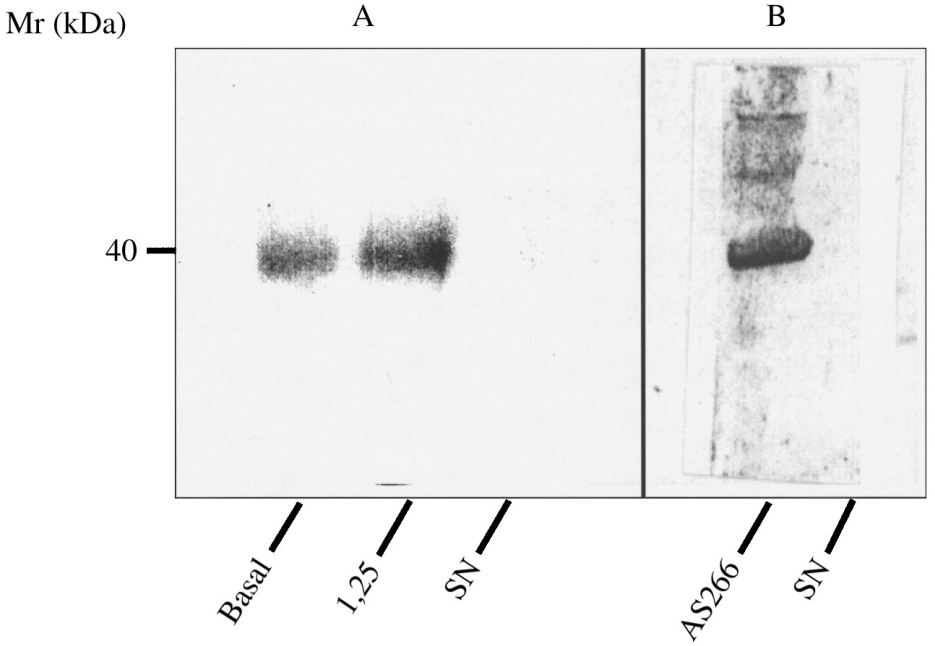

FIG. 3. Effect of $1,25(OH)_2D_3$ on $G_{\alpha i}$ phosphorylation. (A) [^{32}P]-labelled myoblasts were incubated (5 min., 30°C) in the absence (basal) or presence of $1,25(OH)_2D_3$ ("1,25", 1 nM). $G\alpha_i$ was then immunoprecipitated with AS266 antiserum as described (14), resolved by SDS-PAGE (10% acrylamide) and phosphorylation evaluated by autoradiography. Shown is an autoradiogram representative of two independent experiments. SN: preimmune serum. (B) Inmunoblotting of $G\alpha_i$ of myoblast membranes.

relatively modest, and problems are encountered to detect increments in conventional GTPase assays (19). NEM pretreatment of membranes effectively suppresses basal low K_m GTPase activity and inhibits receptor-stimulated GTP hydrolysis by pertussis toxin (PTX)-sensitive G proteins, thereby improving the detection of GTP hydrolysis from agonists coupling to G_s (10, 19). We used this approach to determine if $1,25(OH)_2D_3$ alters G_s function. As shown (Figure 2), no stimulation of low K_m GTPase activity by the hormone was detected in any condition. NEM-treatment effectively unmasked stimulation of GTPase by the G_s-coupled agonist isoproterenol (no detectable in control membranes) and concomitantly abolished the action of the PTX-sensitive G protein activator LPA, thus validating the protocol. This data indicate that G_s stimulation is not mediating AC activation by $1,25(OH)_2D_3$ in these membranes. It was also demonstrated that activation of the AC/cAMP pathway by the sterol effectively couples to PKA activation, in agreement with the PKA inhibitor (Walsh peptide inhibitor)-sensitivity of both the $^{45}Ca^{2+}$ uptake (4) and the rapid increase in the phosphorylation of membrane proteins (Vazquez G, Boland AR and Boland RL, unpublished observations) induced by $1,25(OH)_2D_3$. Here we also demonstrate a rapid increase in $G_{\alpha i}$ phosphorylation in response to $1,25(OH)_2D_3$ treatment, which suggests that this could be, as for some peptide hormones (23), the mechanism by which the sterol exerts its inhibitory action on G_i. At the same time, it represents the first protein to be identified as target for $1,25(OH)_2D_3$-dependent fast membrane protein phosphorylation. In cultured myoblasts, $1,25(OH)_2D_3$-induced activation of PKC elicits a rapid accumulation of cAMP, greatly potentiating cAMP formation induced by the sterol itself (24). If, as in other cell systems (23), $1,25(OH)_2D_3$-dependent $G_{\alpha i}$ phosphorylation in myoblasts is mediated by PKC, is under current investigation.

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