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Effects of Divalent Cations and of a Calcimimetic on Adrenocorticotropic Hormone Release in Pituitary Tumor Cells

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The calcium sensing receptor (CaSR), a member of the G-protein coupled receptor family, is expressed on a variety of cell types and responds to extracellular calcium. We have characterized pharmacological properties of (±)NPS 568, a calcimimetic, toward cloned rat brain extracellular Ca^{2+}-sensing receptor (CaSR) expressed in Chinese hamster ovary (CHO) cells and constitutive mouse CaSR in AtT-20 cells. In the presence of 1.3 mM Ca^{2+}, the calcimimetic displayed a potency in the micromolar range in augmenting the inositol phosphates (IP) response in both cell lines and behaved as a full agonist. (±)NPS 568 stimulated formation of arachidonic acid release in CHO(CaSR) with a similar potency. The IP dose response curves of (±)NPS 568 were shifted to the left in the presence of increasing Ca^{2+}, indicating that the potency of the drug is dependent on extracellular Ca^{2+} in both cells. In AtT-20 cells, Ca^{2+} and Ba^{2+}, two CaSR agonists, induced a potent stimulation of adrenocorticotropic hormone (ACTH) secretion. In the presence of 1.8 mM Ca^{2+}, (±)NPS 568 led to a dose dependent secretion of ACTH with an EC_{50} of 0.3 μM and a maximal effect comparable to Ca^{2+}. The similar potency of the calcimimetic on IP and ACTH responses and the sensitivity of these responses to extracellular Ca^{2+} indicate that the Ca^{2+}-sensing receptor expressed in AtT-20 cells is implicated in ACTH release. These data further characterize the pharmacology of the Ca^{2+}-sensing receptor and argue for a role for extracellular Ca^{2+} and CaSRs in controlling ACTH secretion, a hormone implicated in several types of stress. © 1997 Academic Press

Serum Ca^{2+} is tightly regulated by the major calciotropic hormones that are parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3. The extracellular Ca^{2+}-sensing receptor (CaSR) located on the parathyroid cells regulates PTH secretion in response to minor changes of serum Ca^{2+} and plays a key role in serum calcium homeostasis (1, 2). We have identified a homologous CaSR in brain and pituitary of the rat (3). CaSR belongs to the superfamily of G protein coupled receptors and shows a limited homology with the glutamate metabotropic receptor family (1, 3). Cloned CaSR expressed in CHO cells responds to mM concentrations of divalent cations and to various cationic ligands leading to activation of PLC and PLA_2 enzymes (4). In humans, point mutations in CaSR are characterized by slight modifications of the serum Ca^{2+} set point leading to dramatic physiological effects (5).

ACTH, the adrenocorticotropic hormone, is secreted by a restricted population of cells in the anterior pituitary. The hormone exerts its primary effects on the adrenal cortex where it mediates secretion of corticoid hormones. Among other secretagogues, CRF, the corticotropin releasing hormone synthesized in the paraventricular nucleus of the hypothalamus, plays a major role in ACTH release (6). In addition, variation of extracellular Ca^{2+} appears to modulate the secretion of ACTH. Acute hypercalcemia is followed by a several fold increase of serum ACTH levels (7) and variations of serum Ca^{2+} within the physiological range is accompanied by a significant increase of serum ACTH (8). The exact mechanism by which Ca^{2+} influences ACTH secretion is still unknown.

However, extracellular Ca^{2+} has no effect on basal ACTH secretion on cultured rat pituitary cells (9) whereas Ca^{2+}-sensing receptors have been detected in rat pituitary (3, 10) and on bovine anterior pituitary cells (11). AtT-20 cells are derived from an ACTH-secreting cell line from a mouse pituitary tumor. These cells express an endogenous CaSR coupled to PLC stimula-

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tion and cAMP production (10). We report here the pharmacological properties of (±)NPS 568, a compound proposed as a calcimimetic (12), on cloned rat CaSR (3) and on mouse CaSR expressed constitutively in AtT-20 cells. Interestingly, Ca^{2+}, Ba^{2+} and (±)NPS 568 were found to be potent secretagogues of ACTH release in AtT-20 cells.

EXPERIMENTAL PROCEDURES

Cell culture. CHO(CaSR) and CHO (WT*) cells are Chinese hamster ovary cells transfected or not, respectively, with the rat Ca^{2+}-sensing receptor and have been described (4). These cells were cultured in basal Ham's F-12 medium (0.3 mM Ca^{2+}, 0.6 mM Mg^{2+}) as previously described (4). AtT-20/D16v (Americain Type Culture Collection) (AtT-20) cells were grown in Ham's F-12 medium or in DMEM (1.8 mM Ca^{2+}, 0.6 mM Mg^{2+}), both supplemented with 15% (V/V) horse serum and 2.5% (V/V) fetal calf serum. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

[^{3}H]AA release. CHO(CaSR) were seeded in 24-well plates and cultured overnight in their growth medium containing 0.5 μCi/ml [^{3}H]Arachidonic Acid ([^{3}H]AA) (Dupont NEN). [^{3}H]AA release was performed as described (4) in Ham's F-12 medium supplemented with $CaCl_2$ to a final concentration of 1.3 mM.

[^{3}H]IP formation. Cells, in 24-well plates, were cultured overnight in their growth medium containing 0.5 μCi/ml myo-[^{3}H]inositol ([^{3}H]IP) (Amersham Corp.). Incubation with drugs and isolation of [^{3}H]inositol phosphates were performed (13) as described (4).

ACTH secretion. AtT-20 cells were seeded in 24-well plates and cultured overnight in their growth medium. They were incubated with 1 ml of basal medium alone or supplemented with ions or the indicated drugs for 90 min at 37°C. ACTH release was measured by using a radioimmunoassay (CIS Bio International, France) on supernatant samples which were stored at −70°C.

Generation of polyclonal antisera to rat CaSR. A synthetic peptide KHRNSMRQNSLEAQR based on the rat CaSR sequence (3) was synthesized, conjugated to bovine serum albumin via glutaraldehyde and injected in rabbits to raise antiserum (3). Antibodies were affinity-purified on an ovalbumin-CaSR peptide conjugate immobilized on CNBr activated Sepharose.

Western blot analysis. Cells were homogenized in ice-cold 50 mM Tris.HCl, pH 7.4 containing EDTA (1 mM), aprotinin (10 μg/ml), leupeptin (10 μg/ml), benzamidine (60μg/ml) and centrifuged at 100,000 × g for 1 hr. Pellets were resuspended in buffer and proteins were separated on a 8% polyacrylamide gel, transferred to nitrocellulose membranes, probed overnight with affinity-purified antibodies (1 μg/ml) and developed with enhanced chemiluminescence (ECL, Amersham). For preabsorption experiments, the antibodies were preincubated overnight at 4° C with excess CaSR peptide (20 μg/ml).

Northern blot analysis. Total RNA was isolated from AtT-20 cells cultured in Ham's F-12 medium. Poly(A)$^+$ RNA was further purified by oligo(dT) cellulose purification (Boehringer). For Northern blot analyses, 2μg of Poly(A)$^+$ mRNAs were electrophoresed on a 1.3 % agarose as previously described (3) and blotted onto a nylon filter (Hybond N$^+$, Amersham) and subjected to Northern blot analysis as described (3). A DNA probe ^{32}P-labeled by nick-translation and corresponding to the nucleotide sequence encoding amino acid 175-923 of the rat CaSR protein (3) was used. Blots were washed in 0.2 × SSC/0.1 % SDS for two 20-min periods at 42° C and for two 10-min periods at 55° C, then exposed to film for 1 day at −80° C.

Drugs. Ions (chloride form) and Corticotropin Releasing Hormone (CRF) were from Sigma. (±)NPS 568 was synthesized in the hydrochloride form (RHD). This drug was dissolved in water and stored at −20 °C at 10 mM. Other drugs were from Boehringer Mannheim. Data are expressed as means ± S.E.M. of at least triplicate experiments and varied less than 5% in any given experiment. Statistical significance was assessed by the Mann-Whitney-Wilcoxon test. $p < 0.01$ was considered significant.

RESULTS

CHO(CaSR) cells stably expressing rat CaSR have been used as a model for evaluating CaSR pharmacology (4). We first investigated the ability of the calcimimetic (±)NPS 568 (12) to stimulate [^{3}H]IP accumulation and [^{3}H]AA release in these cells. Experiments were performed in Ham's F-12 medium supplemented with 1.3 mM Ca^{2+}, a concentration which does not activate either response in these cells (4). (±)NPS 568 elicits a five-fold increase of [^{3}H]IP response with an EC_{50} of 3.9 ± 0.8 μM and a maximal effect observed at 30 μM (Fig. 1A). The maximal effect represents 135 ± 10 % that of 10 mM Ca^{2+} on [^{3}H]IP response (data from ten independent experiments). (±)NPS 568 does not stimulate [^{3}H]IP response in CHO(WT*) cells below 30 μM (Fig. 1A). In CHO(CaSR) and under similar experimental conditions, (±)NPS 568 induces a five-fold increase of [^{3}H]AA release over basal level (Fig. 1B) with an EC_{50} of 1.6 ± 0.4 μM. The maximal effect of this response is comparable to that observed with ATP (300 μM), acting on a constitutive purinergic receptor (data not shown), and represents 50 % that obtained with the calcium ionophore A23187 (2 μM) (Fig. 1B). Comparison of the maximal effect of (±)NPS 568 and Ca^{2+} on [^{3}H]AA is not possible since Ca^{2+} directly increases phospholipase A_2 (PLA_2) activity (4).

AtT-20 cells were labeled with myo-[^{3}H]inositol and the formation of [^{3}H]IP in response to Ca^{2+}, Mg^{2+}, Ba^{2+} and (±)NPS 568 was monitored. Divalent cations elicit a pronounced [^{3}H]IP response in AtT-20 cells (Fig. 2A and Fig. 2B). When these cells are cultured in Ham's F-12 medium, increasing Ca^{2+} from 0.3 mM to 2 mM leads to a 1.8-fold increase of [^{3}H]IP response. Further analysis of these data leads to an EC_{50} of 1.3 mM. Augmenting Ca^{2+} from 2 mM to 8 mM is followed by a further 5-7 fold increase of the [^{3}H]IP response, a response showing marked cooperativity (n_H = 4.1) and an EC_{50} of 4.1 mM (Fig. 2A). This dose-response curve analysis is further corroborated by a Hill plot of the data which indicates two components (Fig. 2A inset). In DMEM, which contains 1.8 mM Ca^{2+}, increasing Mg^{2+} from 0.6 mM to 8 mM results in a 50 % maximal increase of [^{3}H]IP accumulation compared to Ca^{2+} (Fig. 2B). The curve shows a shallow concentration response (EC_{50} of 6 mM) and an n_H of 3. Thus, as observed in CHO(CaSR) (4), Mg^{2+} behaves as a partial agonist for the IP response. Under similar conditions, Ba^{2+} potently stimulates [^{3}H]IP response (EC_{50} of 3 mM) with an 80 % maximal effect compared to Ca^{2+}. In DMEM,

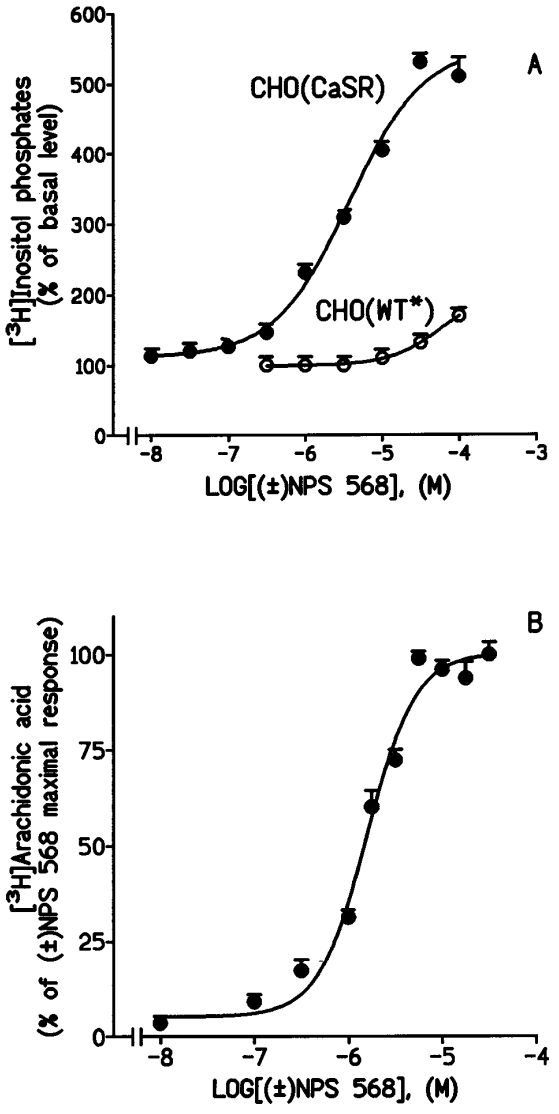

FIG. 1. (±)NPS 568-induced accumulation of [^{3}H]IP and [^{3}H]AA in transfected CHO cells. (A) After prelabeling with myo-[^{3}H]inositol, CHO(WT*), or CHO(CaSR), cells were washed twice with basal Ham's F-12 medium (0.3 mM Ca^{2+}, 0.6 mM Mg^{2+}) supplemented by 10 mM LiCl and incubated for 15 min in the same medium. Medium was removed and cells were incubated for 30 min in the same medium containing 1.3 mM Ca^{2+} alone or increasing (±)NPS 568 concentrations or 10 mM Ca^{2+}. In CHO(CaSR), mean ± S.E.M. of basal and stimulated (10 mM Ca^{2+}) [^{3}H]IP were 125 ± 2 and 581 ± 26 cpm, respectively. [^{3}H]IP basal level (mean ± S.E.M.) was 105 ± 7 cpm in CHO(WT*) and was not different from that obtained with 10 mM Ca^{2+}. (B) CHO(CaSR) cells were prelabeled with [^{3}H]AA, washed twice with 1 ml of basal Ham's F-12 medium supplemented with 0.2% BSA, and incubated 30 min in the same medium containing 1.3 mM Ca^{2+} alone or increasing (±)NPS 568 concentrations. Means ± S.E.M. of basal and stimulated (30 μM (±)NPS 568) [^{3}H]AA were 224 ± 23 and 1072 ± 50 cpm, respectively, or 2734 ± 184 cpm in the presence of 10 mM Ca^{2+}. [^{3}H]AA release induced by A23187 (2μM), a calcium ionophore, was 2216 ± 77 cpm. Data shown in A and B are expressed as % of basal [^{3}H]IP accumulation or of maximal (±)NPS 568 [^{3}H]AA release, respectively, and are representative of three independent experiments performed in triplicate.

(±)NPS 568 displays an EC_{50} of 1.6 ± 0.5 μM in stimulating [^{3}H]IP accumulation in AtT-20 cells (Fig. 2C). The maximal effect obtained at 10 μM (±)NPS 568 (104 ± 10%) is comparable to that of 10 mM Ca^{2+} (Fig. 2C). These results further characterize the expression of a divalent cation sensing receptor linked to PLC activation in these cells (10).

In absence of a suitable labeled ligand, the binding sites of (±)NPS 568 on CaSR have not been identified so far. We evaluated the potency of this drug in AtT-20 and CHO(CaSR) cells, in Ham's F-12 medium containing various Ca^{2+} concentrations (Fig. 3). In CHO (CaSR) cells, raising Ca^{2+} from 0.3 mM to 2.3 mM results in an increase of both maximal response and potency of (±)NPS 568. The concentration of (±)NPS 568 needed to obtain 50% of the [^{3}H]IP response to 10 mM Ca^{2+} was estimated to be 90 μM, 20 μM, 2 μM or 0.5 μM at 0.3 mM, 0.8 mM, 1.3 mM or 2.3 mM Ca^{2+} respectively (Fig. 3A). In AtT-20 cells, [^{3}H]IP response to (±)NPS 568 is also dependent on extracellular Ca^{2+} (Fig. 3B). In basal Ham's F-12 medium (0.3 mM Ca^{2+}), (±)NPS 568 does not significantly stimulate the [^{3}H]IP response. At 2.3 mM Ca^{2+}, which produces a two-fold increase in [^{3}H]IP levels compared to the basal level (determined at 0.3 mM Ca^{2+}), (±)NPS 568 shows an EC_{50} of 0.7 ± 0.1 μM and a maximal effect of 120 ± 3% compared to Ca^{2+}.

We evaluated the effect of CRF (a known secretagogue of ACTH), of various divalent cations and of (±)NPS 568 on ACTH secretion in AtT-20 cells (Fig. 4, Table 1). In DMEM, addition of Ca^{2+} leads to a dose-dependent secretion of ACTH (Fig. 4A). The effect of 6 mM Ca^{2+} was comparable to that of CRF (0.01 μM) used at a concentration corresponding to 80 % of its maximal effect (data not shown). In this medium, the effect of Ba^{2+} on ACTH secretion was already evident at 1 mM, its maximal effect being comparable to that of 5 mM Ca^{2+} (Fig. 4A).

In DMEM, increasing (±)NPS 568 leads to a dose-dependent secretion of ACTH up to 1 μM (Fig. 4B). The maximal effect of the drug is observed in the μM range and an EC_{50} of 0.3 μM is deduced from the curve which shows a strong cooperativity. Above 3 μM (±)NPS 568, the ACTH secretion diminishes and represents only a 20 % increase of basal levels at 10 μM. Interestingly, when the experiment is carried out in Ham's F-12 medium (0.3 mM Ca^{2+}), a 2.5- and 5.3-fold increase of ACTH secretion is produced by elevating Ca^{2+} to 1.8 mM and 7 mM, respectively (Table 1). Under the same conditions, Ba^{2+}(1 mM) induces a two-fold increase of ACTH secretion and its maximal effect, reached at 3 mM, is comparable to that of Ca^{2+} (data not shown). These data indicate that these two divalent cations are potent ACTH secretagogues in AtT-20 cells. The effect of (±)NPS 568 on ACTH secretion appears to be dependent on extracellular Ca^{2+}. In the presence of 0.3 mM

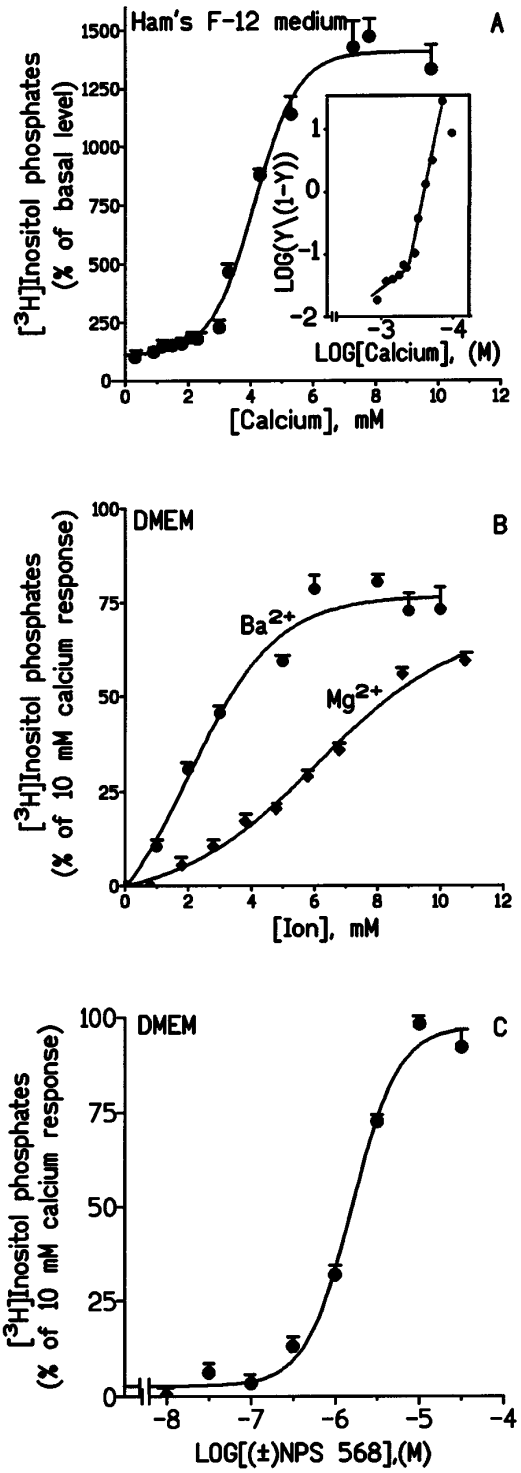

FIG. 2. Calcium, magnesium, barium, and (±)NPS 568-induced accumulation of [^{3}H]IP in AtT-20 cells. (A) AtT-20 cells were prelabeled in basal Ham's F-12 medium supplemented with myo-[^{3}H]-inositol. [^{3}H]IP response to Ca^{2+} was determined as depicted in Fig.1A. Mean ± S.E.M. of basal level [^{3}H]IP was 116 ± 4 cpm. Hill plot and Hill coefficient (n_H) for Ca^{2+} induced accumulation of [^{3}H]IP from AtT-20 cells is indicated as inset. (B and C) AtT-20 cells were prelabeled overnight in DMEM with myo-[^{3}H]inositol, then washed twice with basal DMEM (1.8 mM Ca^{2+}, 0.8 mM Mg^{2+}) supplemented with 10 mM LiCl. [^{3}H]IP response to Ca^{2+}, Mg^{2+}, Ba^{2+}, or (±)NPS 568 was further determined in this medium. Data are expressed as the % of basal in A, or maximal (10 mM Ca^{2+}) response in B and C, and are representative of three to five independent experiments.

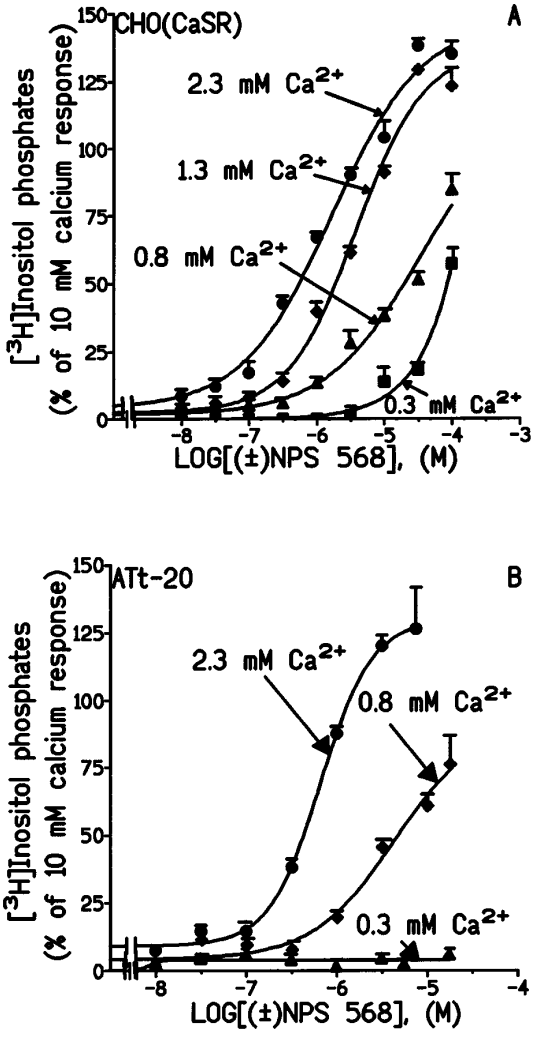

FIG. 3. Effect of calcium on (±)NPS 568-induced accumulation of [^{3}H]IP in CHO(CaSR) and AtT-20 cells. [^{3}H]IP response to (±)NPS 568 was determined for CHO(CaSR) cells (A) or AtT-20 cells (B) in Ham's F-12 medium at the indicated Ca^{2+}. Data shown are expressed as % of maximal (10 mM Ca^{2+}) [^{3}H]IP accumulation over basal level (0.3 mM Ca^{2+}) and are representative of three independent experiments.

Ca^{2+}, the effect of (±)NPS 568 (2 μM) on ACTH secretion represents a 1.3 fold increase over the basal level. In the presence of 1.8 mM Ca^{2+}, the effect of (±)NPS 568 (2 μM) is comparable to that obtained with maximal Ca^{2+} (Table 1, and data not shown).

We investigated the expression of CaSR transcripts and proteins in our AtT-20 cell culture. mRNA from AtT-20 cells cultured in Ham's F-12 medium were sub-

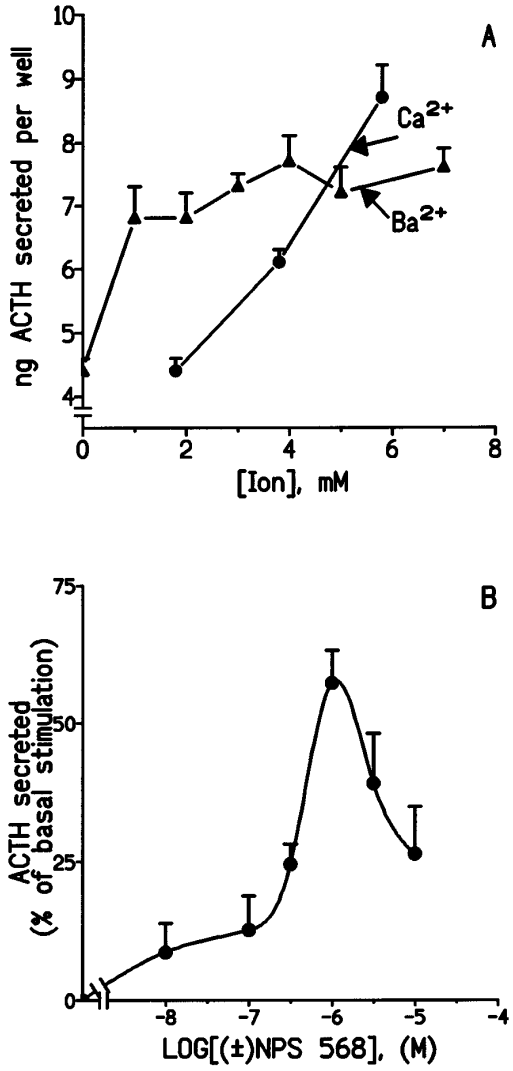

FIG. 4. Effect of calcium, barium, and (±)NPS 568 on ACTH secretion in AtT-20 cells. AtT-20 cells were seeded in 24-well plates and were incubated for 90 min at 37 °C in basal DMEM alone or supplemented with increasing concentrations of Ca^{2+} or Ba^{2+} (A) or (±)NPS 568 (B). In A, ACTH secretion in response to 0.01 μM CRF was 7.0 ± 0.2 ng ACTH per well. Data are expressed as ng ACTH secreted/well (A) or as the percentage of ACTH secretion over basal level (B). Data are representative of a typical experiment over three performed in triplicate (A) or are the mean of three independent experiments (B). (*) Not significantly different from basal level.

jected to Northern blot analysis and probed with a [^{32}P]-labeled probe corresponding to the nucleotide sequence encompassing the amino-terminal tail and the seven putative transmembrane domains of the rat CaSR (3). A major 7.5 kb mRNA transcript and two minor transcripts of 6.5 kb and 5.5 kb were identified (Fig. 5). The expression of a CaSR protein was investigated by immunoblot analysis of membrane extracts of AtT-20 cells cultured in Ham's F-12 medium. A rabbit specific antiserum which is directed against the carboxyl domain of the rat CaSR protein was used. Two immunoreactive bands at 160 kDa and 140 kDa were evidenced in extracts from AtT-20 cells or HEK-293 cells expressing rat CaSR (Fig. 6, lanes 1 and 2). These immunoreactive bands were not present in untransfected HEK-293 cell extracts (data not shown) and were not detected when the antiserum was preabsorbed with the antigen peptide (Fig. 6, lanes 3 and 4).

TABLE 1

ACTH Secretion in Response to Calcium, CRF, and (±)NPS 568 in AtT-20 Cells

[Ca^{2+}], mM	Drug	ACTH, ng/ml mean ± S.E.M.	% of basal level
0.3	None	1.09 ± 0.06	100
0.3	CRF, 0.01 μM	1.79 ± 0.08*	164
0.3	(±)NPS 568, 2 μM	1.40 ± 0.10*	128
1.8	None	2.70 ± 0.17	245
1.8	CRF, 0.01 μM	3.95 ± 0.23	368
1.8	(±)NPS 568, 2 μM	4.58 ± 0.18	420
7	None	5.75 ± 0.30	527

Note. Cells were incubated 90 min with 0.01 μM CRF or 2 μM (±)NPS 568 in basal Ham's F-12 medium containing 0.3 or 1.8 mM Ca^{2+}. Data are from one experiment performed in triplicate repeated with similar results.

(*)Statistically significant compared to basal level ($p < 0.01$) using the Mann-Whitney-Wilcoxon test.

DISCUSSION

The recent cloning of the gene encoding a Ca^{2+}-sensing receptor from bovine parathyroid (1) has led to the

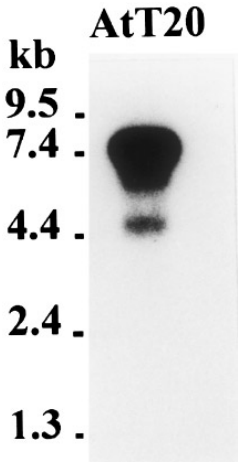

FIG. 5. Northern blot analysis of calcium sensing receptor mRNA in AtT-20 cells. Poly(A)$^+$ RNA (2μg) prepared from AtT-20 cultured in Ham's F-12 medium were submitted to Northern blot analysis using a rat CaSR probe. Molecular sizes of RNA markers (in kb) are indicated. Blot was exposed for one day at −80° C.

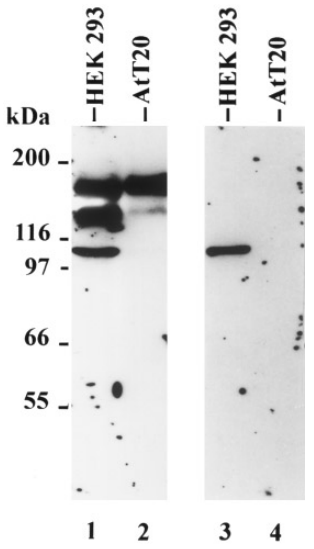

FIG. 6. Immunoblot analysis of membrane extracts from AtT-20 cells or rat CaSR expressing HEK-293 cells. (A and B) Proteins from AtT-20 cells (20μg) or from HEK-293 cells (5μg) transiently expressing rat CaSR were separated on an 8% polyacrylamide gel and electroblotted onto nitro cellulose filters and incubated with a rabbit polyclonal antiserum to a peptide sequence in the C-terminal domain of rat CaSR. A representative blot from three separate experiments is shown. (B) Preabsorption of the antibody with an excess of the antigen peptide (20 μg/ml) completely blocked the 160 and 140 kDa immunoreactive bands. The 100 kDa band observed in HEK-293 is not blocked indicating that the band is not related to CaSR.

identification of the same protein in various tissues including brain (3), kidney (14) and thyroid (15). Expression of CaSR transcripts has also been reported in ileum, lung, testis and pituitary (3, 16) as well as in bone marrow (17). The role of CaSR in some of these tissues is related to calcium homeostasis but remains unclear, particularly in pituitary and brain where we have localized CaSR on nerve terminals (3).

CaSR constitutively expressed in AtT-20 cells is pharmacologically very similar to the cloned rat CaSR expressed in CHO(CaSR) cells as indicated by several observations: i) the IP concentration response curve to Ca^{2+}, Ba^{2+} and Mg^{2+}, all divalent cation CaSR agonists (3), occurs with the same potency in both cells ii) the potencies of (±)NPS 568 on IP responses under similar Ca^{2+} conditions are comparable iii) in both cases these potencies are dependent on extracellular Ca^{2+}. Biochemical and molecular properties of cloned rat CaSR and calcium sensing receptor on AtT-20 cells are also very similar in agreement with the high homology found between the two cDNAs (3, 10). Two peptides of 140 kDa and 160 kDa are evidenced in preparations from HEK-293 cells and may correspond to distinct glycosylation states of the receptor sensitive to N-glycosidase F (data not shown). These observations are in agreement with the molecular weight of CaSR deduced from its amino acid sequence and with the presence of numerous potential glycosylation sites identified in the putative amino-terminal tail of the receptor (3, 4). In AtT-20 cells, a major 160 kDa band is identified and may also correspond to glycosylated forms of the mouse CaSR receptor. The 140 kDa peptide may correspond to a different glycosylated state of the receptor or, alternatively, to a proteolytic product of the 160 kDa peptide. Moreover, Northern blot analysis of RNA prepared from AtT-20 cells identified a major 7.5 kb transcript previously detected in this cell line (10).

AtT-20 cells secrete ACTH in response to various secretagogues (18, 19) and this secretion is also dependent on extracellular Ca^{2+} (19, 20). However, the link with a Ca^{2+}-sensing receptor has not been established so far. In the present studies, Ca^{2+}, but also Ba^{2+}, both CaSR agonists, potently stimulate ACTH secretion.

Modulation of ACTH secretion by CaSR in AtT-20 cells is further confirmed by the effect of (±)NPS 568. Its potency on ACTH secretion is observed in the micromolar range, in good agreement with its potency on IP response obtained in AtT-20 cells or in CHO(CaSR) cells using similar basal Ca^{2+} levels. At concentrations higher than 3 μM, the ACTH response curve to (±)NPS 568 is characterized by an apparent decrease of ACTH secretion. The reasons for these observations are not yet clear since no apparent cell toxicity was evidenced at 10 μM (±)NPS 568 as assessed by the trypan blue exclusion test (data not shown).

However, at low Ca^{2+} (0.3 mM), (±)NPS 568 (2 μM) has only a limited effect on ACTH secretion whereas its effect is clearly potentiated in the presence of higher Ca^{2+}, in agreement with pharmacological data obtained from IP response. The cell machinery involved in ACTH secretion is functional at low Ca^{2+} since response to CRF was easily detectable (Table 1). Indeed, a low Ca^{2+} level is also sufficient for PLC activation as indicated by the IP response observed to ATP (300 μm) in CHO(WT*) acting on constitutive purinergic receptors. This response was similar in the presence of 0.3 mM or 2.3 mM Ca^{2+} (data not shown). Thus, the potency of (±)NPS 568 or of related drugs on CaSR responses should be more pronounced in physiological and pathophysiological conditions where extracellular Ca^{2+} levels will be elevated. The calcimimetic may bind on CaSR at a different site than Ca^{2+}. Calcium presumably interacts with clusters of negatively charged aspartate or glutamate residues located in the amino-tail of the receptor (1, 3). The site of interaction of (±)NPS 568 with CaSR has not been precisely established. It may bind at the level of the hydrophobic segment formed by the seven putative transmembrane domains (21) and presumably involves an allosteric regulatory site.

Interestingly, Ca^{2+} from 0.3 mM to 1.8 mM leads to a 2- to -3 fold increase in basal ACTH secretion which

should be compared to the significant IP response observed in this Ca^{2+} range, presumably resulting from the activation of higher affinity Ca^{2+} binding sites. Moreover, increasing Ca^{2+} from 0.3 to 2.3 mM for 24 h in the culture medium of AtT-20 cells leads to a 1.8-fold increase in the level of CaSR mRNA, an effect attributed to CaSR activation (10). CaSR antagonists, when designed, should be helpful to clarify such responses associated to CaSR activation by extracellular calcium. CaSR functions as a Ca^{2+}-sensing receptor *in vivo* and probably also as a Mg^{2+} sensor, a cation found at comparable concentration in serum (2). This is also suggested by the similar potency of Ca^{2+} and Mg^{2+} in stimulating IP response in CHO(CaSR) cells (4) or AtT-20 cells (Fig. 2B). Several hypercalcemia states due to genetic mutations of CaSR (22-24) are also characterized by alteration of Mg^{2+} homeostasis with hypermagnesemia (25).

In our studies, ACTH secretion in AtT-20 cells presumably involved second messengers generated by the activation of PLC. However, the participation of the cAMP cascade is not excluded since stimulation of cAMP production has been shown to occur through CaSR activation in AtT-20 cells (10).

ACTH, an opiomelanocortin peptide, is secreted from anterior pituitary corticotroph cells (6) and CRF is one of the most potent secretagogues of ACTH secretion and biosynthesis. In AtT-20 cells, Ca^{2+} or a calcimimetic are more potent than CRF in stimulating ACTH secretion suggesting that CaSR activation plays a major role in this effect. CaSR transcripts and proteins have been localized in hypothalamic structures (3, 26) suggesting a possible involvement for CaSR in the control of CRF which is synthesized in the same area before being released in the pituitary zone.

Modulation of CaSR participates in the PTH and calcitonin secretion in parathyroid and thyroid cells, respectively (2) both hormones are implicated in extracellular Ca^{2+} homeostasis. Our work may indicate a role for extracellular Ca^{2+} and Ca^{2+}-sensing receptor in modulating ACTH secretion, a hormone which is induced by multiple physiological and psychological disturbances, particularly in several types of stress. Indeed, alterations of secretion of luteinizing hormone and follicule stimulating hormone, as well as prolactin and thyroid stimulating hormone in response to Ca^{2+} infusion have been reported in humans, but the role of Ca^{2+}-sensing receptors has not been investigated (7, 8, 27, 30). The AtT-20 cell line offers a convenient model to study CaSR pharmacology and regulation of hormone secretion.

In brain, CaSR is located on nerve terminals (3) where extracellular Ca^{2+} controls neurotransmitter release. Brain CaSR presumably responds to local changes of Ca^{2+} levels resulting from neuronal activity (31). However, alterations in the extracellular Ca^{2+} concentrations may activate other proteins than CaSR. Recently, a novel cation channel sensitive to variations of extracellular Ca^{2+} has been characterized in cultured hippocampal neurons (32) and there are evidences for subtypes of calcium sensors in addition to CaSR (2). Characterization of the pharmacological properties of calcimimetics or calcilytics should be helpful to delineate responses associated to alterations of extracellular calcium and related to CaSR activation. Moreover, such ligands, once available, might be useful in the treatment of various endocrine diseases (5) including those associated with pituitary functions.

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