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Spontaneous and Agonist-Induced Calcium Oscillations in Single Human Nonfunctioning Adenoma Cells

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The effects of gonadotropin-releasing hormone (GnRH) and GnRH-associated peptide (GAP) on cytosolic free calcium concentration ($[Ca^{2+}]_i$) were investigated in 20 human nonfunctioning pituitary adenomas. We divided these tumors into three classes according to their response pattern to hypothalamic peptides. In type I adenomas (8 out of 20 adenomas), GnRH and GAP mobilized intracellular calcium ions stored in a thapsigargin (TG)-sensitive store. For the same concentration of agonist, two distinct patterns of GnRH-, GAP-induced Ca^{2+} mobilization were observed (1) sinusoidal oscillations, and (2) monophasic transient. The latter is followed by a protein kinase C (PKC)-dependent increase in calcium influx through L-type channels. In type II adenomas (7 out of 20 adenomas), GnRH and GAP only stimulate calcium influx through dihydropyridine-sensitive Ca^{2+} channels by a PKC-dependent mechanism. TG (1 μ*M*) did not affect $[Ca^{2+}]_i$ in these cells, suggesting that they do not possess TG-sensitive Ca^{2+} pools. All the effects of GnRH and GAP were blocked by an inhibitor of phospholipase C (PLC), suggesting that they were owing to the activation of the phosphoinositide turnover. Type I and type II adenoma cells showed spontaneous Ca^{2+} oscillations that were blocked by dihydropyridines and inhibition of PKC activity. GnRH and GAP had no effect on the $[Ca^{2+}]_i$ of type III adenoma cells that were also characterized by a low resting $[Ca^{2+}]_i$ and by the absence of spontaneous Ca^{2+} fluctuations. K^+-induced depolarization provoked a reduced Ca^{2+} influx, whereas TG had no effect on the $[Ca^{2+}]_i$ of type III adenoma cells. The variety of $[Ca^{2+}]_i$ response patterns makes these cells a good cell model for studying calcium homeostasis in pituitary cells.

Key Words: Microspectrofluorimetry; gonadotrophin-releasing hormone; gonadotrophin-releasing hormone-associated peptide; pKCs; IP3; dihydropyridines; thapsigargin; Ca^{2+} influx; intracellular Ca^{2+} stores.

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Introduction

"Nonfunctioning" adenomas of the pituitary are so called because they do not cause any specific symptoms resulting from hormone overproduction. However, studies have shown that most "silent" adenomas are composed of identifiable cell types that have characteristic immunoreactivity for specific hormones. Moreover, many "silent" adenomas have been reported to secrete luteinizing hormone (LH), follicle stimulating hormone (FSH), and/or free α subunits of pituitary glycoprotein hormones in vitro (Ridgway, 1984; Snyder, 1985; Lamberts et al., 1987).

"Silent" adenoma cells possess functional receptors for several hypothalamic peptides, such as thyreoliberin (Le Dafniet et al., 1987), dopamine (Bevan and Burke, 1986), somatostatin (Ikuyama et al., 1985), and gonadotropin-releasing hormone (GnRH) (Spada et al., 1991). These receptors are coupled to several signal transduction pathways that activate intracellular effector generation (Ca^{2+}, IP3, DG, cAMP, and so on). Thus, these cells provide a good model for studying the effects of secretagogs on human tumor gonadotrophs.

In this study, we investigated and compared the effects of the GnRH and GnRH-associated peptide (GAP) on the intracellular calcium concentration ($[Ca^{2+}]_i$) of individual human "nonfunctioning" adenoma cells, using the calcium-sensitive fluorescent probe, Indo 1. GAP is a 56 amino acid peptide occupying the carboxy-terminal region of the GnRH precursor. It was found to be a potent inhibitor of prolactin secretion and to stimulate the release of gonadotropins in rat pituitary cell cultures (Nikolics et al., 1985).

Recently, we have shown that GAP inhibits prolactin (PRL) release from GH3 cells (Vacher et al., 1991), normal rat lactotrophs (Vacher et al., 1991), and human prolactinoma cells (Vacher et al., 1991; Dufy-Barbe et al., 1993) through coordinated action on voltage-dependent K^+ and Ca^{2+} conductances, and on cAMP production (Vacher et al., 1991; Tran Van Chuoï et al., 1993). This study suggests that GAP may activate another second messenger system, probably the phosphatidylinositol-phospholipase C pathway, also triggered by GnRH in gonadotrophs. In addition, we provide evidence that GAP and GnRH act through dis-

Table 1

Basal $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ Responses to GnRH and GAP

Type	Spontaneous activity	Basal [Ca2+]i	GnRH responsive cells			GAP responsive cells		
			0.1 n*M*	1 n*M*	10 n*M*	0.1 n*M*	1 n*M*	10 n*M*
I	33%	142 ± 7 n*M*	24%	35%[a]; 17%[b]	47%[a]; 28%[b]	0%	40%[a]; 15%[b]	52%[a]; 23%[b]
	(*n* = 273)	(*n* = 199)	(*n* = 50)	(*n* = 72)	(*n* = 106)	(*n* = 31)	(*n* = 66)	(*n* = 106)
II	66%	146 ± 9 n*M*	25%	62%	84%	35%	55%	80%
	(*n* = 153)	(*n* = 52)	(*n* = 44)	(*n* = 38)	(*n* = 29)	(*n* = 25)	(*n* = 31)	(*n* = 40)
III	0%	102 ± 8 n*M*	0%	0%	0%	0%	0%	0%
	(*n* = 75)	(*n* = 75)	(*n* = 75)	(*n* = 12)	(*n* = 13)	(*n* = 16)	(*n* = 12)	(*n* = 12)

n, number of cells tested; mean ± SD.

[a]Percentage of cells responding to GAP or GnRH by sinusoidal $[Ca^{2+}]_i$ oscillations.

[b]Percentage of cells responding to GAP or GnRH by a biphasic $[Ca^{2+}]_i$ increase.

tinct receptors. Our results confirm the great heterogeneity of the calcium response to secretagogs in pituitary cells. For the same adenoma, the same type of cell (FSH- or LH-secreting cell), the same concentration of agonist, the same day of experimentation, and the same Petri dish, we observed several types of monophasic or sinusoidal $[Ca^{2+}]_i$ oscillation responses to GAP or GnRH. The response also depends on the adenoma. We have separated the adenomas into three classes, based on their calcium response.

Results

Characterization of Several Types of Spontaneous Ca^{2+} Oscillations in Nonsecreting Adenoma Cells

In the Material and Methods section, we describe three types of adenoma (type I to III) according to their response to GnRH and GAP. These adenomas also had different resting $[Ca^{2+}]_i$ levels.

Two main types of resting $[Ca^{2+}]_i$ were recorded in single human nonsecreting adenoma cells (Table 1). Some cells were silent (type I: 67%, $n = 273$; type II: 34%, $n = 153$; type III: 100%, $n = 75$). In such cells, basal $[Ca^{2+}]_i$ was not significantly different in types I and II (about 145 n*M*), but was much lower in type III (about 102 n*M*). Application of low Ca^{2+}-containing HBSS (*see* Materials and Methods, Fig.1Aa) or of Ca^{2+} channel inhibitors, such as dihydropyridines (PN 200-110; Fig. 1Ba) slightly reduced the $[Ca^{2+}]_i$ in type I and II adenoma silent cells (Fig. 1 Aa, Ba). This effect suggests the involvement of L-type Ca^{2+} channels in regulating resting $[Ca^{2+}]_i$ independently of their effect in triggering off the spontaneous Ca^{2+} oscillations associated with action potentials. Inhibition of calcium entry was without effect on the basal $[Ca^{2+}]_i$ of type III adenomas (data not shown).

In many cells, however, we observed spontaneous Ca^{2+} oscillations of various amplitudes and frequencies (Fig. 1). In type I adenomas, two categories of spontaneous Ca^{2+} oscillations were observed: (1) small fluctuations (50–100 n*M*), recorded in 19 out of 73 cells, disappeared under

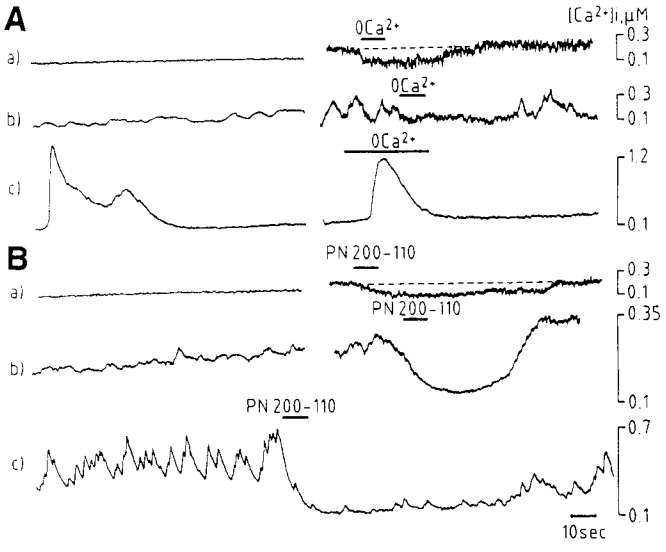

Fig. 1. Spontaneous $[Ca^{2+}]_i$ oscillations in single human "nonfunctioning" adenoma cells. LH- and FSH-containing cells were identified by immunocytochemistry as described in the Materials and Methods section. We classified the adenomas in two categories: type I **(A)** and type II **(B)**. A. Resting patterns $[Ca^{2+}]_i$ in type I adenomas. **(a)**. The majority of the cells were silent. Omission of Ca^{2+} ions in the external medium slightly reduced $[Ca^{2+}]_i$. **(b)**. Small spontaneous oscillations were observed in some cells. They disappeared when Ca^{2+} ions were omitted in the external medium. **(c)**. In a few cells, large spikes were recorded, even in the absence of extracellular Ca^{2+}. **(B)**. Resting patterns $[Ca^{2+}]_i$ in type II adenomas. **(a)**. Some cells were silent. Inhibition of L-type Ca^{2+} channels with PN200-110 (0.5 μ*M*) reduced resting $[Ca^{2+}]_i$. (**b** and **c**). The majority of the cells showed spontaneous oscillations of small **(b)** or large **(c)** amplitude that were blocked by PN200-110 (0.5 μ*M*).

extracellular Ca^{2+}-deficient conditions (Fig. 1Ab) or in the presence of dihydropyridines (data not shown); and (2) large Ca^{2+} spikes, observed in only 7% of the cells, persisted in the absence of Ca^{2+} in the extracellular medium (Fig. 1 Ac). In type II adenomas, two categories of spontaneous Ca^{2+} oscillations were also observed (Fig. 1 Bb and

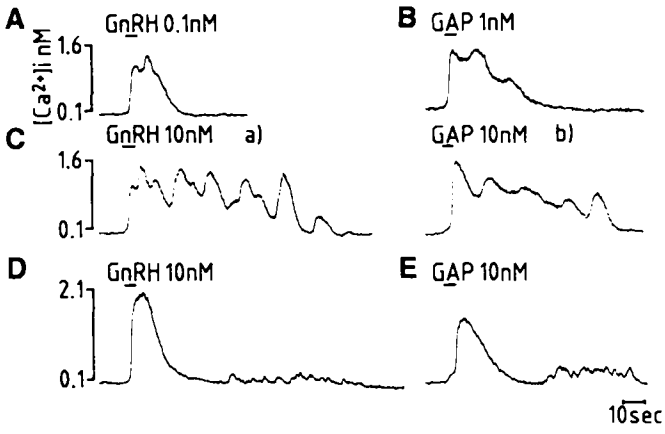

Fig. 2. Effects of GnRH and GAP on the $[Ca^{2+}]_i$ of type I adenoma cells. **(A)** 0.1 n*M* GnRH induced some Ca^{2+} oscillations. **(B)** 1 n*M* GAP has the same effect. **(C)** Higher concentrations (10 n*M*) of GnRH **(a)** and then (2 min later) GAP **(b)** on the same cell resulted in longer responses. (**D** and **E**) Nonsinusoidal responses to GnRH **(D)** and GAP **(E)** were observed in some cells. These biphasic increases in $[Ca^{2+}]_i$ were characterized by a large transient followed by a stimulation of the frequency of Ca^{2+} fluctuations.

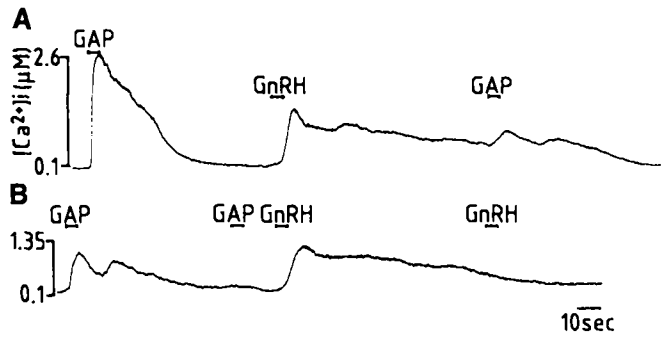

Fig. 3. Effects of successive applications of GnRH (1 n*M*) and GAP (1 n*M*). Recordings of type I adenoma cells. **(A)** Stimulation with different hormones (GAP then GnRH then GAP) at frequent intervals did not desensitize the cell, even though the amplitude of the response often decreased with the number of ejections. **(B)** Rapidly repeated stimulation with the same hormone (GAP and then GAP, or GnRH and then GnRH) desensitized the cell, whereas it remained sensitive to the other hormone.

Bc). They could be distinguished by the amplitude of the Ca^{2+} transients, 50–100 n*M* (Fig. 1 Bb) in 10 out of 53 cells and 200–400 n*M* (Fig. 1Bc) in 25 out of 53 cells, but not by their sensitivity to dihydropyridines, since they were both blocked by PN 200-110. Thus, type II adenomas show Ca^{2+} transients dependent on Ca^{2+} entry through L-type Ca^{2+} channels, whereas type I adenomas also exhibit mobilization of calcium ions stored in intracellular compartments. The high frequency of the calcium transients made it impossible to determine the resting $[Ca^{2+}]_i$ of spontaneously active cells.

GAP and GnRH Induce an Increase in Intracellular Free Calcium in Human Tumor Gonadotrophs

The effects of GAP and GnRH on $[Ca^{2+}]_i$ are summarized in Table 1. In 24% (n = 50) of type I adenoma cells, short-term stimulation with 0.1 n*M* GnRH evoked some sinusoidal $[Ca^{2+}]_i$ oscillations (amplitude: 1490 ± 110 n*M*, n = 12; Fig. 2A). Higher agonist concentrations (1 and 10 n*M*) increased response duration (0.1 n*M*: 28 ± 2.5 s, n = 12; 1 n*M*: 64 ± 11 s, n = 25; 10 n*M*: 112 ± 23 s, n = 50) and the number of responding cells (1 n*M*: 35%, n = 72; 10 n*M*: 47%, n = 106) without any significant effect on response amplitude (1 n*M*: 1535 ± 130 n*M*, n = 25; 10 n*M*: 1570 ± 145 n*M*, n = 50; Fig. 2Ca). Note that $[Ca^{2+}]_i$ did not return to the basal level between spikes during the oscillatory period. The presence of spontaneous fluctuations in $[Ca^{2+}]_i$ did not significantly change the ability of GnRH to evoke $[Ca^{2+}]_i$ responses. These fluctuations disappeared during the oscillatory response to GnRH and then reappeared a few seconds later. At these concentrations, (1 and 10 n*M*) we observed another type of $[Ca^{2+}]_i$ profile in 17% (n = 72, 1 n*M*) and 28% (n = 106, 10 n*M*) of the cells. This response was characterized by a biphasic increase in $[Ca^{2+}]_i$, a large transient (1 n*M* GnRH: 1530 ± 120 n*M*, n = 12; 10 n*M* GnRH: 1995 ± 155 n*M*, n = 30) followed by the triggering of small fluctuations in quiescent cells (Fig. 2D) or by an increase in the frequency of these fluctuations in spontaneously active cells. At 100 p*M*, no response was observed.

Figure 2B shows that 1 n*M* GAP has a similar effect to that of 0.1 n*M* GnRH on $[Ca^{2+}]_i$ (1510 ± 95 n*M*, n = 26). Higher concentrations (10 n*M*) also characterized increased response duration (1 n*M*: 42 ± 5 s, n = 26; 10 n*M*: 73 ± 6 s, n = 54) in 52% of the cells (n = 106, Fig. 2 Cb), but no increase in response amplitude (1450 ± 120 n*M*, n = 55). GAP (10 n*M*) also induced a biphasic effect on $[Ca^{2+}]_i$ in 23% (n = 106, Fig. 2E). The amplitude of the large spike (first phase) was slightly smaller (1345 ± 125 n*M*, n = 24) than that of the 10 n*M* GnRH-induced spike. Some cells (12 out of 76) responded only to GnRH, others (15 out of 76 cells) only to GAP, but the majority (49 out of 76 cells) responded to both GnRH and GAP (Figs. 2C and 3). Two ejections at close intervals (60–80 s) of different agonists (GnRH then GAP or GAP then GnRH) provoked an increase in $[Ca^{2+}]_i$ (Fig. 3A and B), whereas rapidly repeated ejections of the same agonist were ineffective (Fig. 3B). This data suggest that GnRH and GAP act through distinct receptors.

In 25% of the type II adenoma cells, 0.1 n*M* GnRH elicited Ca^{2+} transients in silent cells (Fig. 4A) or raised $[Ca^{2+}]_i$ (185 ± 55 n*M*; n = 11) by increasing the frequency and/or the amplitude of spontaneous Ca^{2+} transients in spontaneously active cells (data not shown). Higher concentrations resulted in a more pronounced response (1 n*M*: 340 ± 150 n*M*, n = 24, 10 n*M*: 420 ± 125 n*M*, n = 24) in a greater number of cells (Table 1). Calcium response amplitude and duration were increased as shown for GAP in Fig. 5.

GAP had the same effect as GnRH on the $[Ca^{2+}]_i$ of type II adenoma cells, but higher concentrations were required;

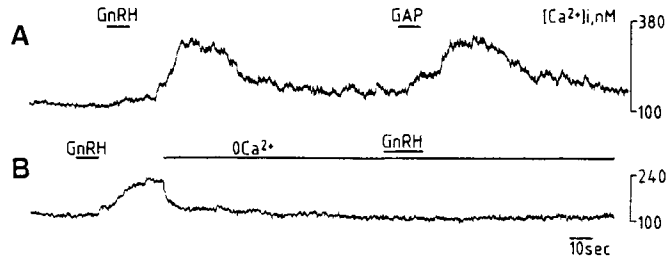

Fig. 4. Effects of GnRH (0.1 n*M*) and GAP (1 n*M*) on the $[Ca^{2+}]_i$ of type II adenoma cells. **(a)** GnRH and GAP increased the amplitude and/or frequency of the Ca^{2+} fluctuations. **(b)** External Ca^{2+} removal completely eliminated any $[Ca^{2+}]_i$ rise on stimulation with 1 n*M* GnRH.

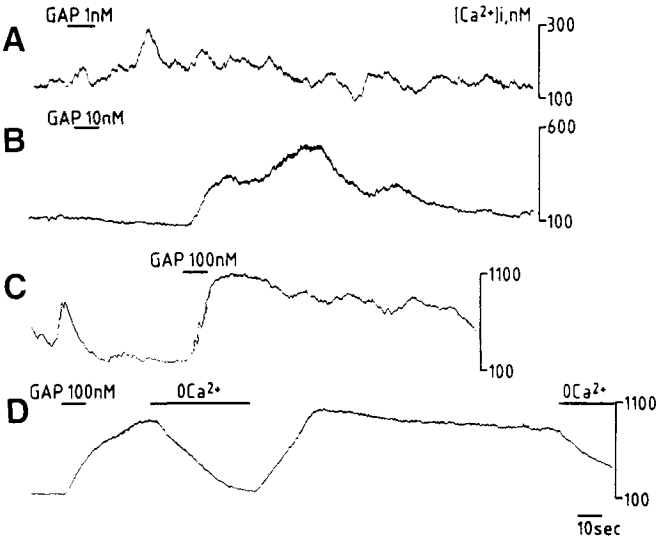

Fig. 5. Dose-dependent effect of GAP on $[Ca^{2+}]_i$ of type II adenoma cells. 1 n*M* **(a)**, 10 n*M* **(b)** and 100 n*M* **(c,d)** GAP, like GnRH, induced a rise in $[Ca^{2+}]_i$. Duration and amplitude of the GAP response increased with concentration. **(d)** External Ca^{2+} removal eliminated GAP-induced Ca^{2+} increase.

0.1 n*M* GAP was ineffective on the $[Ca^{2+}]_i$ of the 25 cells studied. A 10-fold higher concentration induced a delayed, reversible increase in $[Ca^{2+}]_i$ (150 ± 45 n*M*, $n = 34$) in 16 out of 46 cells (Fig. 5A). More pronounced, longer responses were obtained with 10 (380 ± 160 n*M*, $n = 32$) and 100 n*M* (560 ± 195 n*M*, $n = 23$) GAP in silent (Fig. 5B) and spontaneously active (Fig. 5C) cells (10 n*M*: 17 out of 31 cells; 100 n*M*: 32 out of 40 cells). Note that the delay of the response decreases with higher concentrations.

The Relative Roles of Intra- and Extracellular Ca^{2+} in the Response to GnRH and GAP

The different types of response to GAP and GnRH seemed to reflect the involvement of Ca^{2+} from different sources. In order to investigate whether Ca^{2+} entry was involved in GnRH-stimulated $[Ca^{2+}]_i$ responses, the ability of the peptides to trigger $[Ca^{2+}]_i$ responses during a prolonged absence

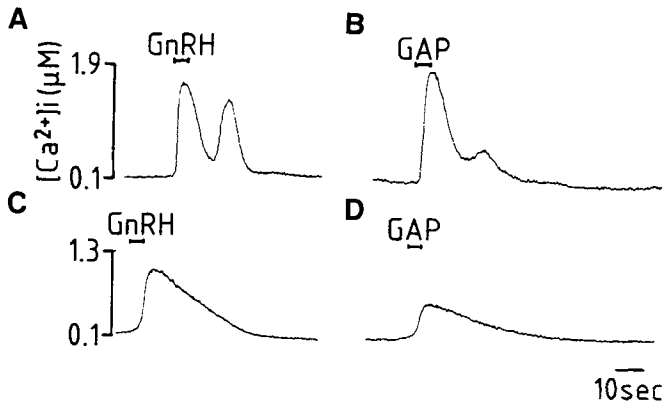

Fig. 6. Extracellular Ca^{2+} dependence of GnRH and GAP-induced Ca^{2+} transients in type I adenoma cells. The type I adenoma cells were bathed in a Ca^{2+}-deprived extracellular medium. Oscillating Ca^{2+} responses to 10 n*M* GnRH **(A)** and 10 n*M* GAP **(B)** were shortened as compared to responses obtained in standard medium (2 m*M* Ca^{2+}, Fig. 2 A, B, and C). **(C, D)**. The nonoscillating Ca^{2+} transient of the biphasic response to GnRH (C) and GAP (D) persisted in the absence of extracellular Ca^{2+}, whereas the second phase disappeared.

of extracellular Ca^{2+} was examined (Fig. 6). The cells were stimulated within 5–20 min of external Ca^{2+} removal, in order to avoid intracellular calcium redistribution.

In type I adenomas, deprivation of extracellular calcium reduced the duration of the GnRH- (control: 107 ± 15 s $n = 15$; $0Ca^{2+}$: 34 ± 12 s, $n = 9$; Fig. 6A) and GAP- (control: 72 ± 11 s, $n = 12$; 0 Ca^{2+}: 35 ± 12 s, $n = 10$; Fig. 6B) induced sinusoidal response. GnRH and GAP response amplitudes were not diminished in the absence of extracellular Ca^{2+} (GnRH, control: 1570 ± 145 n*M*, $n = 50$, 0 Ca^{2+}: 1595 ± 125 n*M*, $n = 13$; GAP, control: 1450 ± 120 n*M*, $n = 55$, 0 Ca^{2+}: 1565 ± 185 n*M*, $n = 11$). Thus, although the $[Ca^{2+}]_i$ responses depend mainly on Ca^{2+} from intracellular stores, Ca^{2+} entry may also participate. This Ca^{2+} influx is probably required to replenish the Ca^{2+} stores that are mobilized and apparently depleted during the initial portion of the response. Dihydropyridines (PN200-110, 0.1–1 μ*M*) did not significantly modify the amplitude or duration of GAP or GnRH response (GnRH, control: 1570 ± 145 n*M*, $n = 50$; 1 μM PN200-110: 1620 ± 125 n*M*, $n = 6$. GAP, control: 1450 ± 120 n*M*, $n = 55$, 1 μ*M* PN200-110: 1495 ± 115 n*M*, $n = 7$). In the case of the biphasic response observed in type I adenoma cells, the absence of extracellular Ca^{2+} or the presence of Ca^{2+} channel inhibitors, such as nickel ions or dihydropyridines, reduced the amplitude of the first large peak (10 n*M* GnRH, control: 1995 ± 155 n*M*, $n = 30$; 0 Ca^{2+}: 1145 ± 85 n*M*, $n = 6$, Fig. 6C; 1 μ*M* PN200-110: 1230 ± 155 n*M*, $n = 7$. 10 n*M* GAP, control: 1345 ± 125, $n = 24$, 0 Ca^{2+}: 645 ± 50 n*M*, $n = 6$, Fig. 6D; 1 μ*M* PN200-110: 765 ± 60 n*M*, $n = 8$) and completely blocked the second phase (small fluctuations). These results suggest that the first phase is, at least partly, dependent on extracellular Ca^{2+}. Though the cells were bathed in Ca^{2+}-deprived medium <20 min before

agonist application, we cannot exclude a partial emptying of intracellular Ca^{2+} stores in a Ca^{2+}-deprived extracellular medium. The major part of Ca^{2+} increase results from mobilization of intracellular Ca^{2+} stores. The secondary rise in $[Ca^{2+}]_i$ represents Ca^{2+} entry.

In type II adenomas, inhibition of Ca^{2+} entry (Ca^{2+}-deprived extracellular medium, or Ca^{2+} channel inhibitors) completely inhibited the Ca^{2+} increases induced by GnRH (Fig. 4B) and GAP (Fig. 5D). Thus, in type II adenomas the two peptides appear to stimulate calcium influx primarily through L-type Ca^{2+} channels (inhibition by dihydropyridines).

Nature of the Second Messengers for Ca^{2+} Influx and for Intracellular Ca^{2+} Mobilization

GnRH has been shown to stimulate the production of both IP3 and diacylglycerol (DG) in rat gonadotrophs. Several studies have suggested that IP3 was responsible for the mobilization of calcium stored in intracellular compartments (endoplasmic reticulum) and DG-protein kinase C (PKC) for the stimulation of Ca^{2+} influx (for review, *see* Naor, 1990). We investigated the involvement of these two pathways in GAP and GnRH action mechanisms in both types of adenomas by several means:

1. Phospholipase C was blocked, using a specific inhibitor, U73122 (Bleasdale et al., 1990; Stojilkovic et al., 1993);
2. The role of IP3 was investigated by depleting the IP3-sensitive Ca^{2+} pools with the nonphorbol tumor promotor thapsigargin (TG) (Thastrup et al., 1990); and
3. The putative involvement of PKCs in GnRH and GAP responses was studied by inhibiting its activity. The cells were incubated with 1 μM phorbol myristate acetate (PMA) for 24 h in order to downregulate PKCs (Ballester and Rosen, 1985).

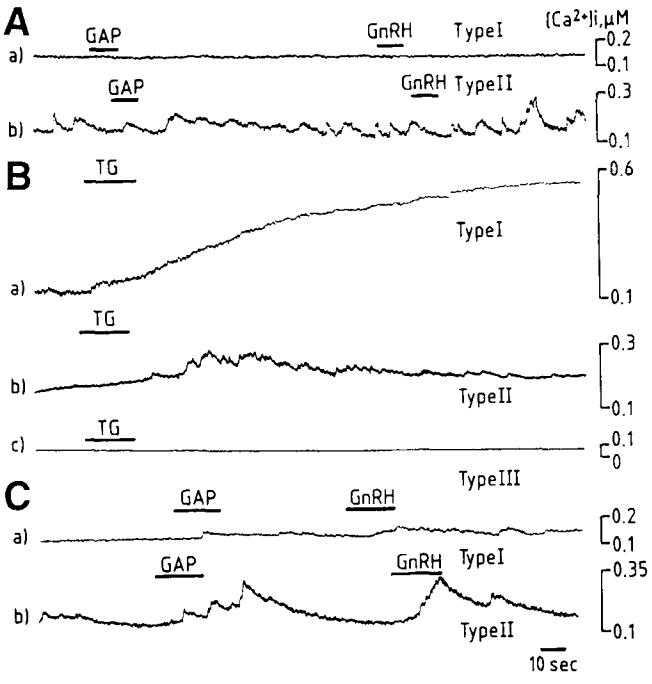

Fig. 7. Involvement of IP3-sensitive Ca^{2+} pools in the response to GAP and GnRH. **(A)** The cells were pretreated with 10 mM U73122, a PLC inhibitor. In these conditions, GAP (10 nM) and GnRH (10 nM) were unable to induce any increase in $[Ca^{2+}]_i$ in type I **(a)** and type II **(b)** cells. **(B) (a)** In type I adenoma cells, TG (1 μM) induced a slow increase in $[Ca^{2+}]_i$. **(b)** In type II adenoma cells, TG (1 μM) did not affect intracellular calcium stores, but stimulated calcium influx in some cells. **(c)** In type III adenoma cells, TG (1 μM)had no effect on $[Ca^{2+}]_i$. **(C) (a)** 10 nM GnRH and 10 nM GAP were applied on type I cells after 10 min of TG treatment (1 μM). This treatment prevented any intracellular calcium mobilization on stimulation with GnRH or GAP. **(b)** In type II adenoma cells, treatment with 1 μM TG for 10 min did not modify the response to GnRH and GAP.

A low concentration (1 μM) of U73122 reduced (type I adenomas: 10 nM GnRH 285 ± 25 nM, n = 6; 10 nM GAP 310 ± 30 nM, n = 5) and a higher concentration (10 μM) completely abolished GnRH- and GAP-induced Ca^{2+} responses in type I and type II adenomas (Fig. 7A). On the other hand, an analog of U73122, U73343 (1–10 μM), with a very weak effect on PLC did not significantly affect the calcium responses to GAP and GnRH in type I (10 nM GAP: 1410 ± 85 nM, n = 14; 10 nM GnRH: 1605 ± 105 nM, n = 18) and type II (10 nM GAP: 355 ± 165 nM, n = 15; 10 nM GnRH: 435 ± 155 nM, n = 14) adenomas.

An acute (10-s) application of 1 μM TG caused an increase in $[Ca^{2+}]_i$ in type I adenomas (675 ± 65 nM, n = 24) but not in type III (n = 28) adenomas (Fig. 7B). In type II adenomas (n = 36) TG elicited Ca^{2+} oscillations in silent cells (Fig. 7Bb) and increased Ca^{2+} oscillation frequency in spontaneously active cells (data not shown). TG effect on type II adenomas disappeared in Ca^{2+}-deprived medium, suggesting that in these cells, TG only stimulated calcium influx. The TG-induced increase in Ca^{2+} was slower and smaller than those induced by GnRH or GAP (Fig. 7Aa). Ten minutes after the addition of TG, $[Ca^{2+}]_i$ returned to basal level. TG preincubation (10 min.) inhibited GnRH- and GAP-induced Ca^{2+} mobilization in type I adenomas (Fig. 7Ca), but did not modify the hormone-induced Ca^{2+} influx in type II adenomas (Fig. 7Cb). This data suggest that GnRH and GAP mobilize intracellular calcium through an IP3-dependent mechanism. Two types of Ca^{2+} mobilization were observed in type I adenomas, oscillating (Fig. 2C) and monophasic (Fig. 2D). Both are related to a TG-sensitive pool. In addition, type II and type III adenoma cells probably do not have IP3-sensitive Ca^{2+} stores, since TG had no effect on these cells (Fig. 7B).

PKC inhibition did not modify the GnRH- or GAP-induced Ca^{2+} mobilization (GnRH, sinusoidal response: control 1570 ± 145 nM, PMA-treated cells 1480 ± 165 nM; nonsinusoidal response: control 1995 ± 155 nM, n = 30, PMA-treated cells 1870 ± 160 nM, n = 7; GAP, sinusoidal response: control 1450 ± 120 nM, n = 55, PMA treated cells 1380 ± 195 nM n = 4, Fig. 8Aa, nonsinusoidal response:

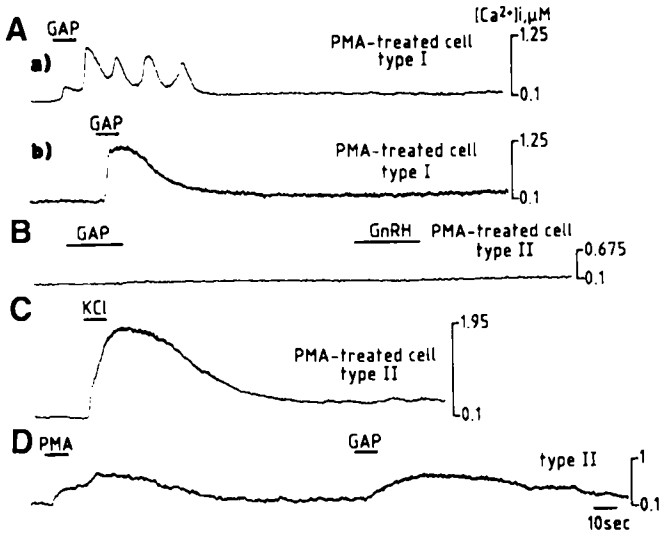

Fig. 8. Involvement of PKC in the response to GnRH and GAP. Cells were treated with 1 μ*M* PMA for 24 h in order to deplete PKC. No spontaneous Ca^{2+} oscillations were observed in the treated cells. **(A) (a)** In type I adenoma cells, the oscillating Ca^{2+} response was not significantly modified by the inhibition of PKC activity. **(b)** Transient mobilization of the biphasic response was unaffected by the treatment, whereas the following stimulation of Ca^{2+} entry was inhibited. **(B)** Downregulation of PKC prevented any $[Ca^{2+}]_i$ rise on stimulation with 10 n*M* GnRH or 10 n*M* GAP. **(C)** Although the treatment completely blocked the spontaneous Ca^{2+} fluctuations and GnRH- or GAP-induced Ca^{2+} entry, 20 m*M* KCl was still able to stimulate Ca^{2+} influx by depolarizing the membrane potential. **(D)** Application of 10 n*M* PMA to type II adenoma cells, like 1 n*M* GAP, stimulated Ca^{2+} entry.

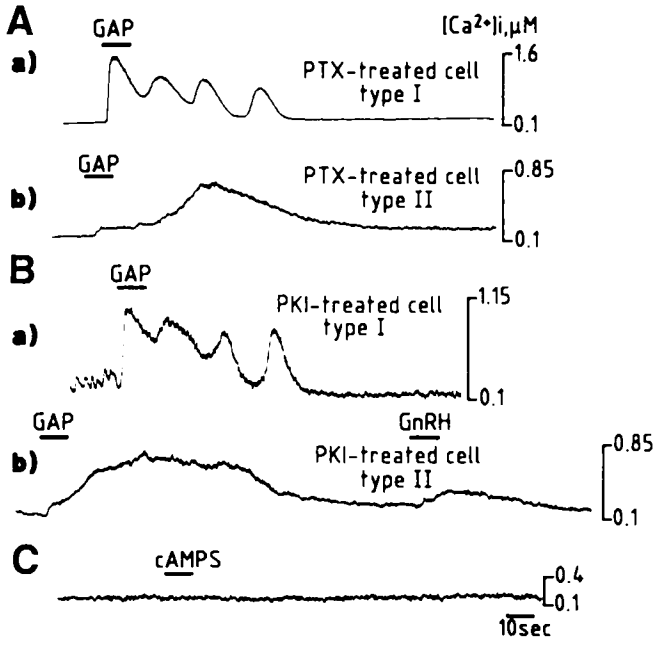

Fig. 9. A PTX-sensitive G protein or the cAMP-pKA pathway did not participate in the GnRH or GAP response. **(A)** Cells were treated with PTX (100 ng/mL) for 10 h in order to inhibit Gi proteins. Such a treatment did not alter the response to GAP (10 n*M*) in type I **(a)** and type II **(b)** adenoma cells. **(B)** Cells were treated with the Rp isomer of cAMPS in order to inhibit pKA. This treatment did not alter the spontaneous Ca^{2+} fluctuations or the response to GAP (10 n*M*) or GnRH (1 n*M*) in type I **(a)** and type II **(b)** adenoma cells. **(C)** Application of the Sp isomer of cAMPS (10 μ*M*), an activator of pKA, was without any effect on the $[Ca^{2+}]_i$ of a type II adenoma cell.

control 1345 ± 125 m*M*, *n* = 24, PMA-treated cells 1320 ± 150 n*M*, *n* = 6, Fig. 8Ab), but completely blocked the spontaneous (type I and type II adenomas) and GAP- or GnRH-elicited (type I and type II adenomas) Ca^{2+} oscillations (18 out of 18 cells) owing to Ca^{2+} influx through L-type Ca^{2+} channels (Fig. 8 Ab and B). Although spontaneous and agonist-induced Ca^{2+} entry were blocked by PMA pretreatment, a depolarization of the cell membrane with 50 m*M* KCl was still able to stimulate Ca^{2+} entry (Fig. 8C). On the other hand, application of 10 n*M* PMA to untreated cells mimicked the action of GnRH or GAP (Fig. 8D) on $[Ca^{2+}]_i$. These data suggest that PKCs control basal and stimulated Ca^{2+} entry in type I and type II adenomas.

Conversely, inhibition or activation of pKC activity had no effect on $[Ca^{2+}]_i$ in type III adenomas. Furthermore, KCl-induced depolarization of the membrane potential provoked only a small increase in $[Ca^{2+}]_i$ (about 100 n*M*) in type III adenoma cells in comparison to the large increase (400–600 n*M*) observed in type II cells. It should be noted that these celis had low basal $[Ca^{2+}]_i$ and no spontaneous transients. Our data suggest that type III adenoma cells possess defective or low density Ca^{2+} channels

We have recently shown that GAP inhibited PRL release in different cell types, normal rat lactotrophs, rat tumor lactotrophs (GH3 cells), and human prolactinoma cells by acting on the pertussis toxin (PTX)-sensitive protein kinase A (pKA) pathway (Vacher et al., 1991; Tran Van Chuoï et al., 1993). Therefore, we investigated the possible involvement of this signal transduction pathway in the GAP and GnRH responses. For this purpose, the cells were treated with PTX (100 ng/mL for 24 h, type I adenomas: 17 cells; type II adenomas: 15 cells) or with the Rp isomer of adenosine-3', 5'-cyclic monophosphothioate (Rp cAMPS, 10 μ*M*, 30–60 min. type I adenomas: 22 cells; type II adenomas: 20 cells), a pKA inhibitor. Neither treatment altered the spontaneous Ca^{2+} oscillations, or the basal $[Ca^{2+}]_i$, nor did they modify the Ca^{2+} responses (entry or release from intracellular stores) to GAP or GnRH in type I (10 n*M* GnRH, sinusoidal response: control 1570 ± 145 n*M*, *n* = 50, PTX-treated cells 1610 ± 175 n*M*, *n* = 5, protein kinase A inhibitor (PKI) or Rp cAMPS-treated cells 1595 ± 155 n*M*, *n* = 6; nonsinusoidal response: control 1995 ± 155 n*M*, *n* = 30, PTX-treated cells 1885 ± 165 n*M*, *n* = 8, PKI-treated cells 1850 ± 180 n*M*, *n* = 5; 10 n*M* GAP, sinusoidal response: control 1450 ± 120 n*M*, *n* = 55, PTX-treated cells 1530 ± 145 n*M*, *n* = 6, Fig. 9Aa, PKI-treated cells 1300 ± 185 n*M*, *n* = 7, Fig. 9Ba, nonsinusoidal response: control 1345 ± 125 n*M*, *n* = 24, PTX-treated

cells 1410 ± 115 n*M*, $n = 4$, PKI-treated cells 1320 ± 95 n*M*, $n = 6$) or type II adenomas (Fig. 9Ab, Bb). In addition, stimulation of PKA with the Sp isomer of cAMPS (10 μM) had no effect on the $[Ca^{2+}]_i$ of type I ($n = 14$ cells) or type II ($n = 21$ cells) adenoma cells (Fig. 9C). These results suggest that the Gi protein-PKA pathway is not involved in the control of basal and GnRH- or GAP-stimulated $[Ca^{2+}]_i$.

Discussion

Nonsecreting adenomas are endocrinologically inactive pituitary tumors. Two-thirds of them, including the cells of the present study, have been shown to secrete or stain immunospecifically for some combination of intact gonadotrophins and/or their subunits. Such cells can thus be considered to belong to the gonadotrophin cell lineage (Bevan and Burke, 1986).

The presence of spontaneous Ca^{2+} fluctuations in rat gonadotrophs is somewhat controversial. Some authors have observed a stable basal $[Ca^{2+}]_i$ in gonadotrophs from ovariectomized adult female Wistar rats (Guérineau et al., 1992). On the other hand, Catt and collaborators (Lida et al., 1991; Stojilkovic et al., 1991) have shown different patterns of spontaneous Ca^{2+} fluctuations in gonadotrophs from ovariectomized adult female Sprague-Dawley rats. The discrepancies between these studies might be explained by the delay between cell preparation and $[Ca^{2+}]_i$ determination, 3 d for Catt's team, and 18–30 h for Mollard's team. Two types of Ca channels have been described in normal rat gonadotrophs (Stutzin et al., 1988; Marchetti et al., 1990) and in αT3-1, a rat cell line of the gonadotroph lineage (Bosma and Hille, 1992): a dihydropyridine-sensitive L-type Ca^{2+} channel and a T-type Ca^{2+} channel. These channels are thought to be activated by PMA (Shangold et al., 1988; Bosma and Hille, 1992). In human gonadotrophs, we have also observed several patterns of spontaneous Ca^{2+} fluctuations (Fig. 1). Recordings were made after 3–10 d of culture. We noted a decrease in the percentage of spontaneously active cells over time. Stable but elevated (about 170 n*M*) basal $[Ca^{2+}]_i$ may be decreased by removing extracellular calcium or by adding dihydropyridines, suggesting a possible calcium influx at the level of the resting membrane potential. Small Ca^{2+} fluctuations (amplitude 10–100 n*M*) were also seen in some cells. These were blocked by dihydropyridines. The third type of Ca^{2+} fluctuations we observed in human gonadotrophs was very similar to the spontaneous transients associated with Ca^{2+}-dependent action potentials described in rat tumor GH3 cells (Schlegel et al., 1987), rat normal lactotrophs (Vacher et al., 1991), and human prolactinoma cells (Vacher et al., 1991; Dufy-Barbe et al., 1993). Furthermore, Ca^{2+}-dependent action potentials were identified in gonadotrophs from female Wistar rats maintained for up to 5 d in culture (Croxton et al., 1988). Both isolated spontaneous transients and bursts were recorded.

Spontaneous Ca^{2+} entry was blocked by dihydropyridines and by downregulation of pKCs. However, pKC inhibition does not block Ca^{2+} channels completely, since a depolarization of the membrane with KCl stimulates calcium influx in pKC-depleted cells. It is speculated that this type of treatment leads to hyperpolarization of the membrane potential below the action potential and spontaneous Ca^{2+} transients' firing threshold. Hyperpolarization of the membrane could be achieved by stimulating a K^+ conductance involved in controlling resting membrane potential. Bosma and Hille (1992) have shown a direct effect of PMA on the L-type Ca^{2+} channel of αT3-1 cells. Another hypothesis for the persistence of the KCl-induced Ca^{2+} influx in pKC-depleted cells is that depolarization may activate a PMA-insensitive Ca^{2+} channel.

GnRH (Hazum and Conn, 1988) and GAP (Nikolics et al., 1985) stimulate the release of LH and FSH gonadotropins from the anterior pituitary. It has been clearly established that this effect of GnRH on gonadotropin secretion in rat cells is calcium-dependent (for review, *see* Conn et al., 1981). GnRH-induced calcium increase derives from two sources: intracellular stores and the extracellular medium (Shangold et al., 1988). The pathway for Ca^{2+} influx appears to be related to the L-type calcium channel (Chang et al., 1986; Schangold et al., 1988; Tse and Hille, 1993), which is thought to be opened by pKC-dependent phosphorylation of the channel (Bosma and Hille, 1992). pKC is activated by DGs produced by the action of phospholipase C at the same time as IP3 is made, after the binding of GnRH to its receptor. GnRH-induced intracellular Ca^{2+} mobilization is likely to be related to IP3 production (Naor, 1990). Two main patterns of mobilization (Guerineau et al., 1992; Stojilkovic and Catt, 1992) were observed in rat gonadotrophs according to the concentration of GnRH used: (1) for low concentrations (0.1–10 n*M*), an oscillating $[Ca^{2+}]_i$ mode characterized by discrete transients spiking from a constant resting level (baseline spiking) was shown in the majority of the cells (about 95%). In the remaining gonadotrophs, sinusoidal spiking was observed, composed of larger and slower oscillations superimposed on an elevated calcium level (Stojilkovic and Catt, 1992). (2) Higher concentrations (100 n*M*) evoked a transient/oscillating $[Ca^{2+}]_i$ mode (Stojilkovic and Catt, 1992). In human gonadotrophs, we did not observe the transient/oscillating response mode, even at high concentrations of GnRH However, in this study, we show two patterns of GnRH-induced calcium release: (1) a sinusoidal spiking mode similar to that observed in some (about 5%) rat gonadotrophs (Stojilkovic and Catt, 1992); and (2) a large monophasic transient followed by a return to the prestimulated basal $[Ca^{2+}]_i$, then by a stimulation of calcium influx. The latter is similar to the Ca^{2+} mobilization induced by TRH (Mollard et al., 1988b, 1990) or GHRP-6 (Bresson-Bépoldin and Dufy-Barbe, 1994) described in lactotrophs and somatotrophs, respectively. Both patterns were observed

with the same concentration of GnRH in cells from the same adenoma. They were both inhibited by a phospholipase C inhibitor (U73122) or by TG, suggesting that they were both caused by the activation of PLC and the subsequent production of IP3. Thus, IP3 is able to produce two types of calcium mobilization, oscillating or nonoscillating, in the same cell type from the same tissue. Human nonfunctioning adenoma cells would be a good model for studying the intracellular biochemical events underlying the oscillatory or nonoscillatory mode of calcium signaling.

Several studies have shown that pKC also plays a role in intracellular calcium mobilization, but its effects vary depending on cell type (for review, *see* Berridge, 1990). In human gonadotrophs, pKC does not participate in the oscillating or monophasic Ca^{2+} responses, since this is not modified by the downregulation of enzyme activity.

To our knowledge, the mechanism by which GAP stimulates gonadotropin release has not been described. In this study, we show that GAP is capable of mobilizing intracellular Ca^{2+} stored in an IP3-sensitive compartment, in the same way as GnRH. This effect of GAP is independent of GnRH receptors, since GAP is able to increase $[Ca^{2+}]_i$ during stimulation with GnRH. The majority of the cells only responded to one of the agonists (GnRH or GAP), and a small percentage of the cells responded to both GnRH and GAP. GAP showed a definite preference for stimulating FSH secretion (Nikolics et al., 1985). It can be speculated that GAP-sensitive cells are FSH-secreting cells, GnRH-sensitive cells are LH-secreting, and GnRH- and GAP-sensitive cells are FSH- and LH-secreting. Nikolics et al. (1985) suggested that a differential release of GnRH and GAP from hypothalamic neurons may change the ratio of circulating gonadotropins observed in certain physiological states.

In type II adenomas, GAP and GnRH were unable to mobilize intracellular calcium. Both peptides only stimulated Ca^{2+} influx through L-type Ca^{2+} channels via a pKC-dependent pathway. Recently, using electrophysiological techniques, we have shown that GnRH stimulated Ca^{2+} entry through L-type voltage-activated Ca^{2+} channels in type II adenomas and that pKC regulates this mechanism, as well as basal ion channel activity (Prévarskaya et al., 1994). IP3-mobilizable Ca^{2+} stores were reduced in these cells, as TG released low quantities of calcium ions. Thus, it can be postulated that, in these particular cells, the binding of GnRH or GAP to their receptors does not mobilize intracellular Ca^{2+} because of the absence of normal calcium pools, probably owing to an alteration in reticulum endoplasmic Ca^{2+} ATPase pumps. However, we cannot exclude that type II adenoma cells were less sensitive to TG than type I. Unlike human cancers, alterations in signal transduction are rarely observed in human pituitary tumors. The only mutations identified to date in pituitary tumors are the G_s α-subunit mutations that cause constitutive activation of the cAMP pathway (Landis et al., 1989), the *ras* mutations that modify cell proliferation and differentiation (Karga et al., 1992), and a point mutation of α-pKC in invasive human pituitary tumors (Alvaro et al., 1993). The G_s α mutations appear to be restricted to tumors of somatotroph origin and are found in only one-third of these tumors. A *ras* mutation was identified in one of the 19 patients (prolactinoma) reported by Karga's team. It will be of interest to determine whether mutations may be responsible for the discrepancies we have observed between type I and type II adenoma, and if so, what types of mutations may be involved.

Previous studies have shown that PTX-sensitive guanylnucleotide-binding proteins (G proteins) do not participate in the Ca^{2+} response to GnRH in rat gonadotrophs (Naor, 1990). On the other hand, we have shown that GAP inhibits PRL release by lactotrophs through a PTX-sensitive pathway (Tran Van Chuoï et al., 1993). Therefore, we studied the effects of GnRH and GAP on $[Ca^{2+}]_i$ in PTX-treated type I and type II human adenoma cells. No modification of the responses to either hormone was observed, suggesting that both peptides act on $[Ca^{2+}]_i$ independently of Gi, Go, and Gp proteins. The treatment consisted of 100 ng/mL PTX for 24 h, which has been shown to be effective in internalizing the toxin and its action on G proteins in rat pituitary cells (Mollard et al., 1988a). Recently, Hawes et al. (1993) demonstrated that PTX treatment reduced GnRH-provoked IP production in rat pituitary cells. This observation was made on total IP population and cannot, therefore, be specifically addressed to the restricted number of IP known to mobilize intracellular Ca^{2+}, such as Ins(1,4,5)P3.

The involvement of cAMP in the GnRH action mechanism is somewhat controversial (for review, *see* Naor, 1990). cAMP may be involved in GnRH-induced gonadotropin biosynthesis (Starzec et al., 1989). GAP-induced inhibition of PRL release in GH3 pituitary cell line is associated with a decrease in cAMP production (Tran Van Chuoï et al., 1993). In human gonadotrophs, inhibition of pKA does not modify the Ca^{2+} responses to GnRH and GAP, suggesting that the cAMP pathway is not activated. Furthermore, activation of pKA has no effect on $[Ca^{2+}]_i$ in type I and type II adenoma cells: neither intracellular Ca^{2+} mobilization nor Ca^{2+} channel activation was observed.

This study suggests that human "nonfunctioning" adenoma cells may be a good cell model for studying Ca^{2+} homeostasis in gonadotrophs. They show several types of spontaneous Ca^{2+} fluctuations and several types of agonist-induced Ca^{2+} mobilization. Our observations indicate the presence of a number of different cell populations, although human pituitary adenomas are thought to represent a clonally derived population of adenomatous cells of gonadotroph origin. Thus, this study confirms the previously proposed hypothesis that gonadotrophs form a number of functionally and morphologically heterogeneous populations in the anterior pituitary (Denef et al., 1980; Lloyd and Childs, 1988; Lewis et al., 1989).They are con-

trolled by a wide spectrum of hypothalamic peptides that are known to act on $[Ca^{2+}]_i$ by different means. For example, we show for the first time an effect of GAP that could be related to the previously described GAP-stimulation of gonadotropin release. Interestingly, this effect of GAP on $[Ca^{2+}]_i$ is similar to that of GnRH and completely opposite of the effect of GAP on lactotrophs. Electrophysiological characterization of ionic conductances of these human tumoral cells is in progress in our laboratory. Further investigations of this cell model may lead to a better understanding of the nature of transduction mechanism(s) in agonist-stimulated gonadotrophs.

Materials and Methods

Human "Nonfunctioning" Pituitary Adenoma Cells

Subjects

The tumors used in our study were issued from 18 patients of both sexes. Diagnosis of clinically nonsecreting pituitary macroadenomas was based on computed tomographic scanning and absence of characteristic clinical syndrome, such as hyperthyroidism, acromegaly, hyperprolactinemia, or Cushing's syndrome.

Cell Culture

These cells were cultured as previously described (Mollard et al., 1988a). Briefly, tumor fragments were obtained by transphenoïdal surgery. The tissue was rinsed several times in a Ca^{2+}- and Mg^{2+} -free Hank's balanced salt solution (HBSS) containing 0.3% bovine serum albumin (BSA, fraction V), then minced and digested with Ca^{2+}-, Mg^{2+}-free HBSS containing 0.2% collagenase (Boehringer, Mannheim, Germany) and 0.3% BSA for 30 min at 37°C. The tumor cells were rinsed and resuspended in complete HBSS containing 0.01% deoxyribonuclease (DNase, type I, Sigma). Final dispersion was achieved by repeated passage through a Pasteur pipet with a fire-polished tip. Most cells were recovered as individual intact elements that excluded the nonvital dye trypan blue. Cells divided slowly and were maintained at least for 3 wk at 37°C in a humidified atmosphere gassed with 95% air–5% CO_2.

For microspectrofluorimetry, the cells were plated on 30-mm-diameter glass coverslides coated with polyornithine (5 mg), and cultured in MEM/F12 (50/50) supplemented by 10% heat-inactivated fetal bovine serum (FBS, Seromed, Strasbourg, France), 8 m*M* $NaHCO_3$, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate for 3–7 d. Half the medium was changed every 2 d. The recordings were routinely performed 3–7 d after setting up the culture.

Immunocytochemical characterization of cells was performed after 2 d of culture as described previously (Mollard et al., 1988; Vacher et al., 1991; Dufy-Barbe et al., 1993). In some experiments, a pertussis toxin (PT) or a phorbol myristate acetate (PMA) treatment was used prior to $[Ca^{2+}]_i$ measurements. In these cases PT (100 ng/mL, List Biologicals, Campbell, CA) and PMA (1 μ*M*, Sigma) were added to the culture medium for 12 and 24 h, respectively, prior to the experiments.

Our data distinguished three types of adenoma according to their calcium response to GnRH or to GAP:

1. Type I: mobilization of stored Ca^{2+} and stimulation of Ca^{2+} entry, patients 1–7;
2. Type II: stimulation of Ca^{2+} entry only, patients 8–13;
3. Type III: no response, patients 14–18.

None of the clinical characteristics (sex, age, treatment, tumor size, and so on) correlated with this classification.

Measurements of Cytosolic-Free Ca^{2+} Concentration in Individual Pituitary Cells

These experiments were performed using the fluorescent probe Indo1 as already described (Vacher et al., 1991) Before each microspectrofluorimetric experiment, the nutrient medium was replaced by a modified HBSS containing 142.6 m*M* NaCl, 5.6 m*M* KCl, 2 m*M* $CaCl_2$, 0.8 m*M* $MgCl_2$, 5 m*M* glucose, and 10 m*M* HEPES, buffered to pH 7.3 with NaOH. The cells were loaded with Indo-1 by exposure to 5 μ*M* Indo-1 acetoxymethyl ester (Indo-1/AM, Calbiochem, Paris, France) and 0.02% (w/v Pluronic F127 (Molecular Probes, Eugene, OR) in HBSS for 20 min at 20°C. We assume that Indo-1 is largely cytoplasmic since the fluorescence appeared to be homogeneously distributed throughout the cells, and no brightly fluorescent spots were observed. Coverslips bearing loaded cells were placed in the chamber of a heated microscope stage and maintained at 37°C. $[Ca^{2+}]_i$ was estimated from Indo-1 fluorescence by the ratio method, using dual wavelength emission (405 and 480 nm) recorded by two separate photometers (Nikon-France, Paris, France). $[Ca^{2+}]_i$ levels were calculated on-line using an analog circuit, which converted the emission fluorescence ratio (F405/F480) to Ca^{2+} concentration according to the formula derived from Grynkiewicz et al. 1985). Ca^{2+} calibrations were obtained under simultaneous whole cell clamp and microspectrofluorimetric measurements. The patch pipets were filled with internal solution containing 10 m*M* EGTA (solution A). 10 m*M* $CaCl_2$, (solution B), or 9.2 m*M* EGTA and 5.4 m*M* $CaCl_2$ (solution C). Solutions A and B allowed the estimation of minimum and maximum values R_{min} and R_{max}, respectively, whereas solution C allowed an evaluation of the product of K_d and β. The latter solution had a calculated free Ca^{2+} of 300 n*M*. R_{min}, R_{max} and K_d.β averaged 0.041 ± 0.01 (n = 12), 068 ± 0.08 (n = 15), and 576 ± 24 n*M* (n = 12), respectively. Linear $[Ca^{2+}]_i$ signals were displayed on a pen recorder (model 2400S, Gould, Paris, France).

Test Substances

Test compounds were applied by pressure ejection from an external micropipet (tip diameter, 2–5 μm), the tip of which was positioned within 10–20 μm of the recorded cell. The concentrations reported are those in the pressure pipet.

GAP (human, 1–56), donated by Dr. Nikolics, was synthesized as previously described (Nikolics et al., 1985). GnRH was purchased from UCB-Bioproducts (Brussels, Belgium). Both peptides were diluted from a concentrated stock solution in the bathing medium before use. U73122 and U73343 were obtained from RBI (Natick, MA). They were first dissolved in dimethyl sulfoxide and, before used, diluted to desired concentration in an appropriate medium.

To obtain a Ca^{2+}-free solution, 2 m*M* EGTA replaced $CaCl_2$ in modified HBSS.

Statistics

Results are expressed as mean ± standard deviation. Statistical comparisons between groups were made by using one-way ANOVA and Fisher PLSD as post-tests, Student's *t*-test was used to compare means when appropriate. Differences with $p < 0.05$ were considered significant. Traces shown in figures are representative of the data obtained in several adenomas of the same type. Each substance was tested on several batches of cells and after different lengths of time in culture.

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