Biochemical Pharmacology, Vol. 43, No. 3, pp. 513–517, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

CORTISOL SECRETION INDUCED BY SUBSTANCE P FROM BOVINE ADRENOCORTICAL CELLS AND ITS INHIBITION BY CALMODULIN INHIBITORS

TOMOKAZU YOSHIDA, MITSUNOBU MIO and KENJI TASAKA*
Department of Pharmacology, Faculty of Pharmaceutical Sciences, Okayama University, Okayama, Japan 700

(*Received* 29 *July* 1991; *accepted* 7 *October* 1991)

Abstract—When primary cultured bovine adrenocortical cells were treated with substance P (SP) at concentrations higher than 10 pM, cortisol output increased in a dose-dependent fashion. Although other neurokinins, such as neurokinin A (NKA) and neurokinin B (NKB), were also effective in secreting cortisol, SP was the most potent among the tested neurokinins, the potency order being SP > NKA>> NKB. This suggests that the NK-1 type receptor on adrenocortical cells may be the site of action of SP on cortisol secretion. The maximal response in SP-induced cortisol secretion was comparable to that elicited by adrenocorticotropic hormone (ACTH). SP-induced cortisol secretion was dependent upon extracellular Ca^{2+} concentrations, and $^{45}Ca^{2+}$ uptake into adrenocortical cells treated with SP was long-lasting. While, in the case of ACTH, $^{45}Ca^{2+}$ uptake proceeded transiently, the increase in intracellular cAMP content was much greater compared with that of SP. Although KT-5720, an inhibitor of protein kinase A, inhibited potently ACTH-induced cortisol secretion, SP-induced secretin was not affected by this inhibitor at all. On the other hand, calmodulin inhibitors, such as calmidazolium, trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, were not more effective in inhibiting SP-induced cortisol secretion than secretion induced by ACTH. The present study indicates that SP may be one of the physiological stimulants of cortisol secretion and that an increase in intracellular Ca^{2+} concentration and the subsequent activation of calmodulin may precede SP-induced cortisol secretion.

It is known that glucocorticoids are secreted from the adrenal cortex treated with adrenocorticotropic hormone (ACTH†) [1] but glucocorticoids are also secreted in response to various polypeptides such as vasoactive intestinal polypeptide [2] and interleukin-1β [3]. Although neurokinins play some important roles as non-adrenergic, non-cholinergic neurotransmitters in many tissues [4] under various physiological or pathophysiological conditions, it is not known whether neurokinins are in some way related to glucocorticoid secretion or not. Furthermore, it has been shown that stimulation of NK-1 receptors may activate the metabolism of phosphoinositides and cause a subsequent increase in intracellular Ca^{2+} concentration [5]. In adrenocortical cells, the corticosteroidogenesis derived from ACTH stimulation is preceded by an activation of Ca^{2+}–calmodulin, diacylglycerol–protein kinase C (C kinase) and/or cAMP–protein kinase A (A kinase) systems [6–8]. However, no decisive evidence has been presented concerning the role of the second messengers in the production and secretion of corticosteroids. In the present study, the effect of SP, a typical NK-1 agonist, on the cortisol output of bovine adrenocortical cells was investigated, focusing on clarification of the intracellular signal transduction in the adrenocortical cells activated by SP.

* Corresponding author. Tel. (81) 862-52-1111 ext. 979; FAX (81) 862-55-7456.

† Abbreviations: SP, substance P; ACTH, adrenocorticotropic hormone; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; HS, horse serum; EGTA, ethyleneglycol-bis-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; db-cAMP, N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate; NKA, neurokinin A; NKB, neurokinin B; A kinase, protein kinase A; C kinase, protein kinase C; TFP, trifluoperazine.

MATERIALS AND METHODS

Preparation of bovine adrenocortical cells. Bovine adrenals were excised immediately after slaughter. After removal of fat tissues, the adrenal cortex was sliced, 0.5–1.0 mm in thickness, using a razor blade on an ice-cold plate. These slices were minced and incubated for 30 min at 37° in 20 mL DMEM supplemented with 3 mM KCl, 1.1 mM $CaCl_2$, 0.2% collagenase Type 1, and 2% bovine serum albumin, under a fixed revolution of 100 rpm. At the end of the incubation period, DMEM containing 10% FCS was added to the reaction mixture and the cells were dispersed by pipetting, repeated more than 10 times. These procedures, both the incubation and dispersion, were repeated once more under the same conditions. Thereafter, the cell suspension was filtered through stainless steel meshes, firstly 200 mesh and then 400 mesh, and the dispersed cells were washed three times with DMEM. The cells were resuspended in DMEM supplemented with 10% FCS, 5% HS, 100 U/mL penicillin and 100 μg/mL streptomycin, and were cultured primarily for 24–72 hr at 37° in a CO_2 incubator with 5% CO_2 in humidified air. Approximately 5×10^7 adrenocortical cells/g tissue were collected and the cell viability, as

assessed by 0.3% Trypan blue dye exclusion test, was higher than 90%. The viable cells were incubated for a further 24 hr in DMEM containing 10% FCS and 5% HS before the experiments.

Measurement of cortisol output. The cultured adrenocortical cells were washed in serum-free DMEM and preincubated for 120 min at 37° in a 24-well plastic plate. The cell density in each well was adjusted to 10^5 cells/mL/well. Thereafter, SP, NKA, NKB or ACTH dissolved in serum-free DMEM at various concentrations was added to each well, and incubation was continued for 60 min at 37°. In some cases, Ca^{2+}-free DMEM containing 20 mM HEPES (pH 7.4) and 1.0 mM EGTA was used to clarify the effect of extracellular Ca^{2+}. When the effect of A kinase inhibitor or calmodulin inhibitors was studied, the test compound was added to the medium simultaneously with one of these stimulants. The reaction was terminated by the addition of ice-cold DMEM to the medium; the cells were sedimented and the cortisol content of the supernatant was measured by means of a radioimmunossay kit.

Measurement of $^{45}Ca^{2+}$ uptake into adrenocortical cells. Cultured adrenocortical cells (10^6 cells in each well) were incubated first in serum-free DMEM and, then, 0.84 μM of $^{45}CaCl_2$ (18.5 kBq/mL) was added to the medium simultaneously with either 10 nM SP or 100 pM ACTH and incubated at 37° for various periods of time. After the reaction was terminated, the medium was aspirated and the cells were washed three times with ice-cold DMEM and dissolved in a solution containing 0.5 N NaOH. Thereafter, the lysate was neutralized with 0.5 N HCl and the radioactivity was determined by means of a liquid scintillation counter (Aloka, LSC-700).

Measurement of cAMP content in adrenocortical cells. Adrenocortical cells were stimulated with 10 nM SP or 100 pM ACTH and the reaction was terminated by adding ice-cold 10% trichloroacetic acid. After centrifugation at 100 *g* for 10 min at 4°, cAMP content in the supernatant was determined by a radioimmunoassay kit.

Chemicals. The compounds used were as follows (sources are indicated in parentheses): DMEM (Nissui, Tokyo, Japan), collagenase Type 1 (Sigma Chemical Co., St Louis, MO, U.S.A.), bovine serum albumin (Sigma), FCS (Hezelton, Lenaxa, KS, U.S.A.), HS (Hezelton), penicillin (Toyo, Jozo, Shizuoka, Japan), streptomycin (Meiji, Tokyo, Japan), SP (Peptide Institute Inc., Osaka, Japan), NKA (Peptide Institute Inc.), NKB (Peptide Institute Inc.), adrenocorticotropic hormone (Peptide Institute Inc.), cortisol radioimmunoassay kit (Nippon DPC Corp., Tokyo, Japan), $^{45}CaCl_2$ (Amersham International, Amersham, U.K.), cAMP radioimmunoassay kit (Yamasa, Tokyo, Japan), db-cAMP (Sigma), KT-5720 (Kyowa Hakko, Tokyo, Japan), calmidazolium (Janssen, Beerse, Belgium), TFP (Wako Pure Chemicals, Osaka, Japan), W-7 (Sigma). Other chemicals used were all reagent grade and were purchased from commercial sources.

Statistical analysis. A one-way analysis of variance with Dunnett's test was used to determine the statistical significance.

RESULTS

Cortisol secretion from bovine adrenocortical cells induced by SP, NKA, NKB and ACTH is indicated in Fig. 1. As shown in this figure, when bovine adrenocortical cells were stimulated by SP at concentrations higher than 10 pM, cortisol secretion was provoked in a dose-dependent manner. The cortisol output induced by SP reached a plateau at concentrations higher than 10 nM. The extent of cortisol output due to 10 nM of SP was nearly the same as that elicited by 100 pM of ACTH. Although NKA and NKB were also effective in eliciting cortisol secretion, their potencies were rather lower than that of SP. Among these neurokinins, the potency order was SP > NKA>> NKB.

As shown in Fig. 2, cortisol output rapidly

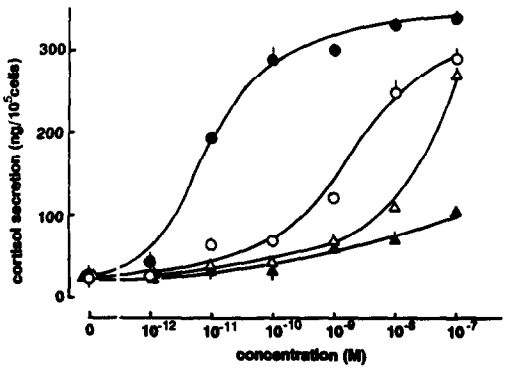

Fig. 1. Cortisol secretion from bovine adrenocortical cells induced by SP, NKA, NKB and ACTH. Each point represents the mean ± SEM obtained from five separate experiments. (N = 5). (○) SP, (△) NKA, (▲) NKB, (●) ACTH. Bovine adrenocortical cells were stimulated with these peptides at 37° for 60 min. Thereafter, cortisol concentrations in the supernatant were measured as described in the text.

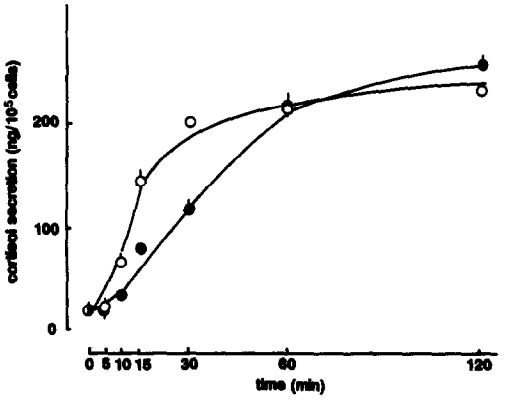

Fig. 2. Time course of cortisol secretion due to SP and ACTH. Each point represents the mean ± SEM obtained from five separate experiments. (○) SP (10 nM), (●) ACTH (100 pM). Bovine adrenocortical cells were stimulated with these peptides at 37° for various periods of time. Thereafter, cortisol concentrations in the supernatant were measured as described in the text.

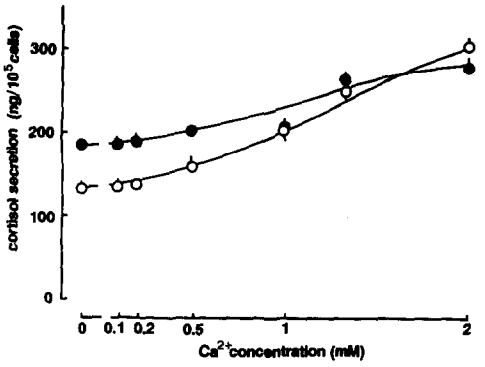

Fig. 3. Effect of extracellular Ca^{2+} concentration on the cortisol secretion from the bovine adrenocortical cells induced by SP and ACTH. Each point represents the mean ± SEM obtained from five separate experiments. (○) SP (10 nM), (●) ACTH (100 pM). Bovine adrenocortical cells were stimulated with these peptides at various concentrations of Ca^{2+} for 60 min at 37°. Thereafter, cortisol concentrations in the supernatant were measured as described in the text.

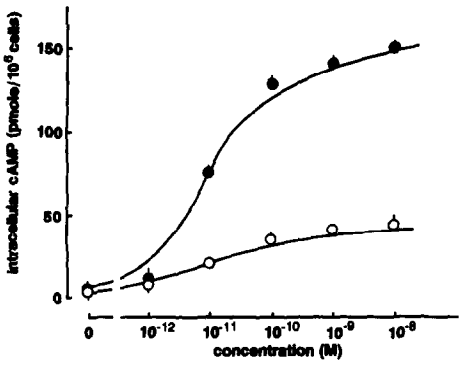

Fig. 4. Changes in intracellular cAMP levels induced by SP and ACTH. Each point indicates the mean ± SEM (N = 6). (○) SP, (●) ACTH. Bovine adrenocortical cells were stimulated with various concentrations of SP and ACTH at 37° for 60 min. Thereafter, cAMP content of the cells was determined as indicated in the text.

increased with SP treatment (10 nM) reaching a plateau within 30 min; almost the same level was maintained for 120 min. SP was much more effective than ACTH in inducing the initial increase in cortisol output.

In order to study the influence of extracellular Ca^{2+} on the cortisol secretion induced by SP, adrenocortical cells were stimulated in a medium containing various concentrations of Ca^{2+} (Fig. 3). In the absence of Ca^{2+} (supplemented with EGTA), SP-induced cortisol output was approximately 49% lower than that determined in the medium containing 2 mM of Ca^{2+}. In contrast, when the effect of ACTH was tested in a Ca^{2+}-free medium, only a 24% reduction in cortisol output was observed. The cortisol secretion due to SP was enhanced by increasing the extracellular Ca^{2+} concentration.

In Fig. 4, the changes in the cAMP content of adrenocortical cells induced by either SP or ACTH are indicated. Both compounds dose-dependently increased the cAMP content of the cells. However, the extent of the cAMP accumulation in the cells induced by SP was much less than of that induced by ACTH. When the sequences of change in cAMP content were compared between SP and ACTH in combination with changes in $^{45}Ca^{2+}$ uptake in relation to cortisol output, SP elicited first an increase in Ca^{2+} uptake and this was followed by a gradual increase in cAMP level. However, in the case of ACTH, both events were conversely related: an abrupt increase in cAMP level was succeeded by a transient increase in Ca^{2+} uptake (Fig. 5). It seems likely that in the stages following the plateau, cAMP plays a more important role than Ca^{2+} in the cortisol secretion induced by ACTH and *vice versa* in SP-induced cortisol secretion. When the cells were treated with db-cAMP 1 hr after stimulation with either SP (10 nM) or ACTH (100 pM), which are capable of eliciting the plateau level secretion of

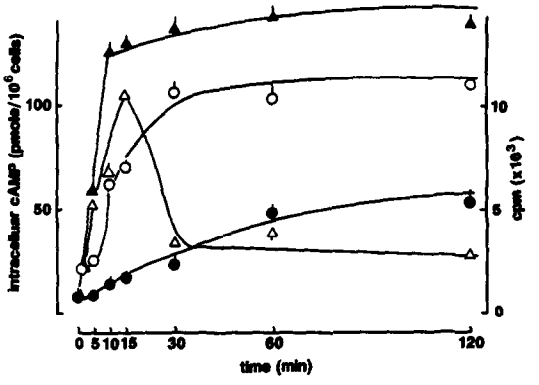

Fig. 5. Time courses of the changes in intracellular cAMP levels and $^{45}Ca^{2+}$ uptake in bovine adrenocortical cells induced by SP (10 nM) and ACTH (100 pM). Each point represents the mean ± SEM obtained from five or six separate experiments. (○) $^{45}Ca^{2+}$ uptake induced by SP, (●) changes in cAMP levels elicited by SP, (△) $^{45}Ca^{2+}$ uptake induced by ACTH, (▲) changes in cAMP levels elicited by ACTH. Bovine adrenocortical cells were stimulated with either SP (10 nM) or ACTH (100 pM) at 37° for various periods of time. Thereafter, cAMP content of and $^{45}Ca^{2+}$ uptake by the cells were determined as indicated in the text.

cortisol, db-cAMP enhanced markedly SP-induced secretion in a dose-dependent fashion, but no such elevation was seen in ACTH-treated cells (Fig. 6).

In order to clarify the role of A kinase in the cortisol output induced by SP and ACTH, various concentrations of KT-5720, an A kinase inhibitor [9], were added to the cells simultaneously with either SP or ACTH (Fig. 7). Although KT-5720 inhibited remarkably and dose-dependently the cortisol output induced by ACTH, SP-induced cortisol secretion was not affected by KT-5720, except at the highest concentration used (50 μM).

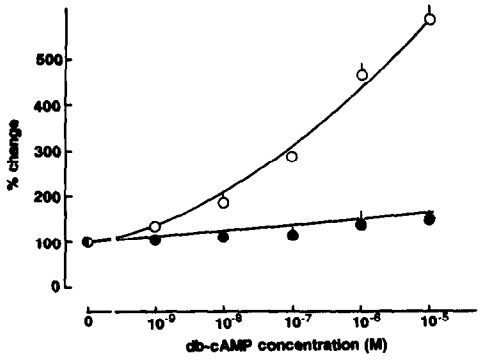

Fig. 6. Additional effect of db-cAMP on the cortisol output of adrenocortical cells previously activated either by SP (10 nM) or ACTH (100 pM). Db-cAMP was added to the medium 1 hr after potent stimulation with SP or ACTH. Thereafter, incubation was continued for another 1 hr. Each point represents the mean ± SEM (N = 5). (○) SP (10 nM), (●) ACTH (100 pM).

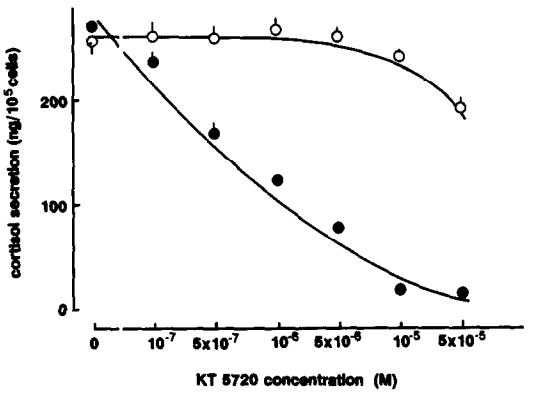

Fig. 7. Effect of KT-5720 on the cortisol secretion elicited by SP (10 nM) and ACTH (100 pM). Each point represents the mean ± SEM (N = 5). (○) SP (10 nM), (●) ACTH (100 pM). KT-5720 was added to the medium simultaneously with either SP or ACTH followed by 60 min of incubation. Thereafter, the corticol output was determined as described in the text.

On the other hand, when calmodulin inhibitors, such as calmidazolium, TFP and W-7, were added to the medium simultaneously with either SP or ACTH, cortisol output induced by these stimulants was inhibited significantly, as indicated in Table 1. The effects of these inhibitors were more evident on the SP-induced secretion than on the secretion induced by ACTH.

DISCUSSION

It is known that glucocorticoids are secreted from adrenocortical cells under physiological stimulation by a variety of substances including not only ACTH but also various peptides such as vasoactive intestinal polypeptide and interleukin-1β [1–3]. Although neurokinins have been indicated as playing important roles as non-adrenergic, non-cholinergic transmitters in various tissues [4], there has been no report regarding the effect of neurokinins on glucocorticoid secretion from the adrenal cortex. The present study shows that neurokinins are effective in eliciting cortisol secretion from bovine adrenocortical cells. Since the effective concentration range and the potency order of neurokinins (SP > NKA>> NKB) are in good agreement with those reported for other tissues [10], it was strongly assumed that the sites of action of neurokinins on the adrenocortical cells correspond to the NK-1 receptor. The effective concentration range of SP in secreting cortisol was close to the K_d value of SP for the NK-1 receptors

Table 1. Inhibitory effects of calmodulin inhibitors on the cortisol secretion of bovine adrenocortical cells induced by SP and ACTH

Compound	Concn (M)	% Inhibition	
		SP (10 nM)	ACTH (100 pM)
Calmidazolium	5×10^{-8}	26.2 ± 2.5	24.8 ± 1.3
	10^{-7}	47.2 ± 3.8*	39.7 ± 0.6
	5×10^{-7}	73.7 ± 0.5†	54.7 ± 3.6
TFP	5×10^{-7}	9.4 ± 2.6*	1.1 ± 0.02
	10^{-6}	34.6 ± 3.4	30.8 ± 0.9
	5×10^{-6}	72.2 ± 4.0†	52.4 ± 1.7
W-7	5×10^{-6}	15.8 ± 0.08	11.9 ± 3.3
	10^{-5}	44.1 ± 2.2†	17.2 ± 3.0
	5×10^{-5}	65.7 ± 4.8†	36.6 ± 2.8

Each compound was added to the medium simultaneously with SP or ACTH, and 60 min of incubation followed.

Each value represents the mean ± SEM of six separate experiments.

* and † represent statistical significance at $P < 0.05$ and $P < 0.01$, respectively, when stimulation by SP was compared with that by ACTH.

in various tissues [10]. This result may suggest that SP, as a potent NK-1 agonist, is also one of the physiological stimuli of glucocorticoid secretion from the adrenal cortex.

It has been shown that in many cells stimulation of the NK-1 receptor induces an increase in intracellular Ca^{2+} concentration and resulting activation of the Ca^{2+}–calmodulin and diacylglycerol–C kinase systems [11, 12]. Since SP-induced cortisol secretion seems to be dependent upon extracellular Ca^{2+} concentrations and SP elicits a sustained $^{45}Ca^{2+}$ uptake, it was suggested that SP-induced stimulation of adrenocortical cells may be triggered by an increased uptake of extracellular Ca^{2+}. However, even in a Ca^{2+}-free medium SP induced cortisol secretion to a level about half of that determined in the presence of extracellular Ca^{2+}. This increase in cortisol secretion may be related to: (1) Ca^{2+} release from intracellular Ca^{2+} store and (2) an increase in intracellular cAMP content elicited by exposure to SP.

In adrenocortical cells, it has been shown that ACTH induces Ca^{2+} uptake and accumulation of cAMP, both of which act cooperatively on glucocorticoid production [13]. As indicated in Fig. 4, SP also causes cAMP accumulation, although the extent is smaller than that elicited by ACTH. Furthermore, when db-cAMP was added to the medium containing adrenocortical cells previously stimulated to the plateau level by either SP or ACTH, SP-induced cortisol secretion was increased further by db-cAMP, but there was no such effect seen in ACTH-treated cells. This may indicate that both cAMP accumulation and the activity of the cAMP-dependent kinase system in association with cortisol production in adrenocortical cells probably reach the maximum when the cells are treated with ACTH (100 pM). In contrast, SP-induced cAMP accumulation was much lower than that elicited by ACTH and this may be the reason for the secondary increase in cortisol output elicited by db-cAMP. Also, this result seems to indicate that db-cAMP is able to enhance the cAMP-dependent pathway in cortisol secretion from cells previously stimulated, leading to the increased production of a second messenger other than cAMP. Although KT-5720, a potent A kinase inhibitor, effectively inhibited ACTH-induced cortisol secretion, SP-induced cortisol secretion was not affected by KT-5720 at all. This may indicate that the cAMP–A kinase system may not be involved in SP-induced cortisol secretion. Based on these findings, it is suggested that SP-induced cortisol secretion is highly dependent upon Ca^{2+} uptake but not upon the intracellular accumulation of cAMP. On the other hand, ACTH-induced cortisol secretion is highly dependent upon cAMP accumulation.

Since the onset of the SP-induced increase in cAMP level was slower than that of Ca^{2+} uptake (Fig. [illegible]), it is supposed that activation of the Ca^{2+}–calmodulin system may induce the subsequent activation of adenylate cyclase, as reported by Salomon [14] in brain membranes. In contrast, calmodulin inhibitors are effective in inhibiting cortisol secretion induced by ACTH and db-cAMP, suggesting that an increase in cAMP level may be the reason for an increase in Ca^{2+} uptake and the subsequent activation of the Ca^{2+}–calmodulin system [15]. When the roles of Ca^{2+} and cAMP in SP- or ACTH-induced cortisol secretion were compared, it was assumed that 'communication' between the Ca^{2+}-calmodulin and cAMP–A kinase systems may take place in the cortisol secretion elicited by either SP or ACTH.

REFERENCES

1. Sala GB, Hayashi K, Catt KJ and Dufau ML, Adrenocorticotropin action in isolated adrenal cells. *J Biol Chem* **254**: 3861–3865, 1979.
2. Leboulenger F, Benyamine M, Delarue C, Netchitailo P, Saint-Pierre S and Vaudry H, Neuronal and paracrine regulation of adrenal steroidogenesis; interactions between acetylcholine, serotonin and vasoactive intestinal peptides on corticosteroid production by frog interrenal tissue. *Brain Res* **453**: 103–109, 1988.
3. Winter JS, Gow KW, Perry YS and Greenberg AH, A stimulatory effect of interleukin-1 on adrenocortical cortisol secretion mediated by prostaglandins. *Endocrinology* **127**: 1904–1909, 1990.
4. Surprenant A, North RA and Katayama Y, Observations on actions of substance P and [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]substance P on single neurons of guinea pig submucous plexus. *Neuroscience* **20**: 189–199, 1987.
5. Merritt J and Rink T, The effects of substance P and carbachol on inositol tris- and tetrakisphosphate formation and cytosolic free calcium in rat parotid acinar cells. *J Biol Chem* **262**: 14912–14916, 1988.
6. Papadopoulos V, Widmaier EP and Hall PF, The role of calmodulin responses to adrenocorticotropin of plasma membranes from adrenal cells. *Endocrinology* **126**: 2465–2473, 1990.
7. Wong M, Rice DA, Parker KL and Schimmer BP, The role of cAMP and cAMP dependent protein kinase in the expression of cholesterol side chain cleavage and steroid 11 β-hydroxylase genes in mouse adrenocortical tumor cells. *J Biol Chem* **264**: 12867–12871, 1989.
8. Culty M, Vilgrain I and Chambaz EM, Steroidogenic properties of phorbol ester and a Ca^{2+}ionophore in bovine adrenocortical cell suspensions. *Biochem Biophys Res Commun* **121**: 499–506, 1984.
9. Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takayashi M, Murakata C, Sato A and Kaneko M, K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent potent kinases. *Biochem Biophys Res Commun* **142**: 436–440, 1987.
10. Regoli D, Drepeau G, Dion S and D'Orleans-Juste P, Receptors for substance P and related neurokinins. *Pharmacology* **38**: 1–15, 1989.
11. Malencik DA and Anderson SR, Peptide cross-linking to calmodulin; attachment of [Tyr^8]substance P. *Biochemistry* **27**: 944–950, 1988.
12. Torrens Y, Daguet De Montety MC, El Etr M, Beaujouan JC and Glowinski J, Tachykinin receptors of the NK1 type (substance P) coupled positively to phospholipase C on cortical astrocytes from the newborn mouse in primary culture. *J Neurochem* **52**: 1913–1918, 1989.
13. Podesta EJ, Milani A, Steffan H and Neher R, Steroidogenic action of calcium ions in isolated adrenocortical cells. *Biochem J* **186**: 391–397, 1980.
14. Harrison JK, Keikilani Henlett GH and Gnegy ME, Regulation of calmodulin-sensitive adenylate cyclase by the stimulatory G-protein, G_s. *J Biol Chem* **264**: 15880–15885, 1989.
15. Hall PH, Osawa S and Thomasson CL, A role for calmodulin in the regulation of steroidogenesis. *J Cell Biol* **90**: 402–407, 1981.