Volume 311, number 1, 41–45 FEBS 11612 October 1992

Okadaic acid and calyculin A enhance the effect of thyrotropin-releasing hormone on GH_3 rat anterior pituitary cells excitability

Luis M. Delgado, Pilar de la Peña, Donato del Camino and Francisco Barros

Departamento de Biología Funcional, Area de Bioquímica, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain

Received 25 June 1992; revised version received 5 August 1992

Thyrotropin-releasing hormone (TRH) causes a transient hyperpolarization followed by several minutes of increased action potential frequency in patch-perforated current-clamped GH_3 cells. Treatment of cells for 5 min with either 2 or 100 nM of the protein phosphatase inhibitor okadaic acid does not affect electrical activity of the cells, but potentiates the enhancement of action potential frequency elicited by a subsequent addition of TRH. Alternatively, 100 nM (but not 2 nM) of okadaic acid added during the second phase of TRH action, further increases the frequency of firing above that produced by the hormone. Similar effects to those of 2 nM okadaic acid are observed with 20 nM calyculin A. These data suggest that a protein phosphatase plays a major role in regulating the delayed effects of TRH on cell excitability in GH_3 cells.

Thyrotropin-releasing hormone; Okadaic acid; Calyculin A; Protein phosphatase; Anterior pituitary; Perforated patch

1. INTRODUCTION

Thyrotropin-releasing hormone (TRH) produces a biphasic effect on the elevation of intracellular Ca^{2+} levels and prolactin secretion in clonal rat anterior pituitary GH_3 cells (reviewed in [1–4]). These two phases are paralleled by changes in cell excitability, consisting of an initial phase of transient hyperpolarization followed by a second phase in which both the rate of production and the length of action (APs) are increased [4–6]. The mechanisms and conductance pathways responsible for the increased production of APs are not well known. It has been suggested that inhibition of various outward currents, including the transient outward current named I_{Kv} and $I_{K(i)}$ [7–9], the voltage- and Ca^{2+}-activated K^+ current [5,10,11], or the inwardly rectifying K^+ current [12,13], is the cause of the second phase of hyperexcitability. It has also been suggested that phosphorylation by protein kinase C (PKC) is involved in modulation of the K^+ channel activity which determines the firing rate [1,2,4,14,15]. However, a direct effect of phosphorylation by the enzyme on a specific K^+ conductance has not been demonstrated in GH_3 cells. It has been proposed that protein phosphatases (PPs)-1 and -2A are the chief enzymes that reverse the actions of PKC [16–18]. These enzymes are inhibited in a potent and selective way by several agents, including okadaic acid (OKA) [18–23], calyculin A (CL-A) [18,21] and tautomycin [22]. Given the very different structures of OKA and CL-A, the combined use of both inhibitors has been regarded as a way to strengthen the evidence that a cellular process is controlled by phosphorylation [18]. We have recently shown that electrical responses to TRH are fully preserved when patch-perforated current-clamped GH_3 cells are studied [5]. On the other hand, recent experiments from our laboratory indicated that treatment of GH_3 cells with OKA specifically potentiates the reductions caused by TRH on inward rectifying K^+ currents [13]. In this report, the effects of OKA and CL-A on electrical activity of the cells are explored. Our results indicate that a phosphorylation–dephosphorylation mechanism may be involved in the regulation of the second phase of TRH action. Furthermore, the possibility that a type 2A protein phosphatase plays a major role in regulating the delayed effects of TRH via its effect on the inward rectifying K^+ current [12,13] is discussed.

2. MATERIALS AND METHODS

Nystatin and TRH were purchased from Sigma (St. Louis, MO, USA). Initial samples of OKA were kindly supplied by Dr. H. Fujiki (National Cancer Center Res. Inst., Tokyo, Japan) and Dr. V. Zitko (Biological Station, St. Andrews, Canada). Subsequently, both OKA and CL-A were purchased from Moana Bioproducts (Honolulu, Hawaii). Maintenance of GH_3 cells (ATCC-CCL 82.1) and conditions of perforated-patch recordings have been described previously [5,13]. Test solutions were applied by continuous perfusion of the 0.2–0.3 ml recording chamber at 1 ml/min. The plotted recordings were obtained at a sampling rate of 10 ms/point except for the APs shown in an expanded time scale in Fig. 1, which were sampled at 0.1 ms/point.

Correspondence address: F. Barros, Departamento de Biología Funcional, Area de Bioquímica, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain. Fax: (34) (8) 5103534.

Abbreviations: TRH, thyrotropin-releasing hormone; AP, action potential; OKA, okadaic acid; CL-A, calyculin A; PKC, protein kinase C; PP, protein phosphatase.

Other Materials and Methods were identical to those described in [5,13]. AP's frequency was determined in periods of at least 80 s either before or 2–5 min after the start of the indicated treatments. Data are expressed as mean ± S.E. for the number of cells indicated in parentheses.

3. RESULTS

We have recently demonstrated that addition of TRH to patch-perforated current-clamped GH_3 cells causes a dose-dependent modification of the electrical activity of these cells [5]. As shown in Fig. 1, this effect resembles that obtained with intracellular microelectrodes, consisting of a transient hyperpolarization after a delay of several seconds, followed by a second phase of sustained increase in AP frequency for several minutes. Most of the cells studied showed the initial hyperpolarization in which the membrane potential increased from a mean resting value of −38.3 ± 0.81 mV (n = 33) to a maximum of −69.0 ± 1.8 mV (n = 33). Furthermore, 32.3 ± 2.5 s (n = 33) were necessary to bring the cells back to the initial value of resting potential. The second phase of increased spiking was elicited in 68% of the cells, but it was not always preceded by a detectable hyperpolarization (see also ref. [5]). The increase in AP frequency averaged 162 ± 16% (n = 21) (see Table I) at the TRH concentration (100 nM) used in this study. As shown in the lower part of Fig. 1, the enhanced rate of firing was generally accompanied by an increase in AP duration, sometimes manifested by the presence of spikes in which the repolarization phase was interrupted by a new depolarization yielding a second overshoot.

The mechanisms and conductance pathways responsible for the increased production of APs are poorly understood. However, phosphorylation by PKC of one or more K^+ channel types has been regarded as a major cause of the enhanced cell excitability in response to TRH (see above). On the other hand, it is well known

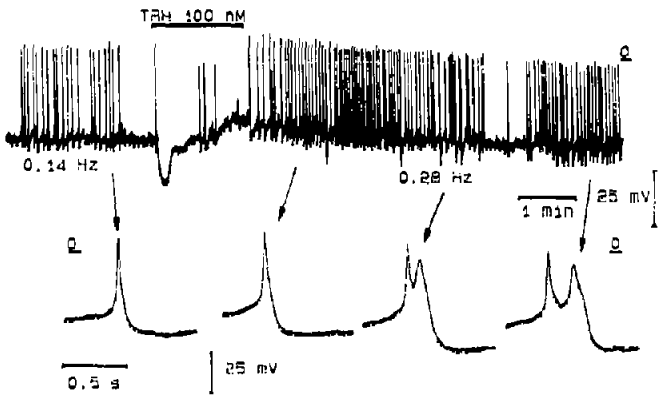

Fig. 1. Effect of TRH on electrical activity in patch-perforated current-clamped GH_3 cells. Application of 100 nM TRH is indicated by a horizontal bar. Four APs obtained at the indicated times are shown in an expanded time scale at the bottom. 0 mV is represented by horizontal lines.

Table 1

Effect of okadaic acid on TRH-induced increases in action potential frequency

Treatment	AP frequency (%)[a]	Number of cells
None	100 (0.23 ± 0.03 Hz)	21
TRH	162 ± 16*	21
OKA 2 nM after TRH	157 ± 8[ns]	12
OKA 100 nM after TRH	261 ± 59**	9
None	100 (0.21 ± 0.04 Hz)	
OKA 2 nM	104 ± 3 (ns)	9
TRH after OKA 2 nM (5 min)	254 ± 27***	
None	100 (0.13 ± 0.03 Hz)	
OKA 100 nM	125 ± 11 (ns)	9
TRH after OKA 100 nM (5 min)	282 ± 46***	

(ns), not significant vs. control before additions.
*, $P < 0.005$ vs. control before additions.
[ns], not significant vs. TRH.
**, $P < 0.025$ vs. TRH.
***, $P < 0.005$ vs. TRH.
[a], Mean ± S.E.M. values are shown. Percentages refer to the same cells before any treatment. Significant values were obtained by a Student's t-test.

that the regulation of a biological process by protein phosphorylation is produced through a dynamic equilibrium between phosphorylation (via protein kinases) and dephosphorylation (via PPs) of the regulatory proteins. Since the delayed responses to TRH are fully preserved under perforated-patch conditions, the effects of OKA and CL-A (two potent and selective inhibitors of PPs 1 and 2A) on the electrical activity of the cells was studied. Fig. 2A shows that treatment of a GH_3 cell with 2 nM OKA for approximately 10 min does not modify the rate of production of APs. The subsequent perfusion of the cell with 100 nM TRH originates a biphasic response similar to that elicited in OKA-untreated cells. However, as stated below, the enhancement of electrical activity during the second phase of TRH action is considerably potentiated by pretreatment with OKA for 5 min (see Table I). Unlike the results obtained by prior treatment with 2 nM OKA, addition of the inhibitor at the same concentration two or three minutes after the second phase of TRH action has started, does not cause any further increase in the frequency of firing (Fig. 2B). The value of 2 nM represents a concentration ca. 2–100 times above the K_i (0.03 to 1 nM, refs. [17–23]) for inhibition of PP-2A by OKA. However, it is clearly lower than the K_i value (10–500 nM, refs. [17–23]) for inhibition of PP-1. The results of similar experiments performed at a concentration of 100 nM OKA are depicted in Fig. 3. Fig. 3A shows that addition of the inhibitor does not modify the frequency of firing, but subsequent perfusion with 100 nM TRH elicits a second phase of enhanced AP frequency which is significantly higher than that produced after treat-

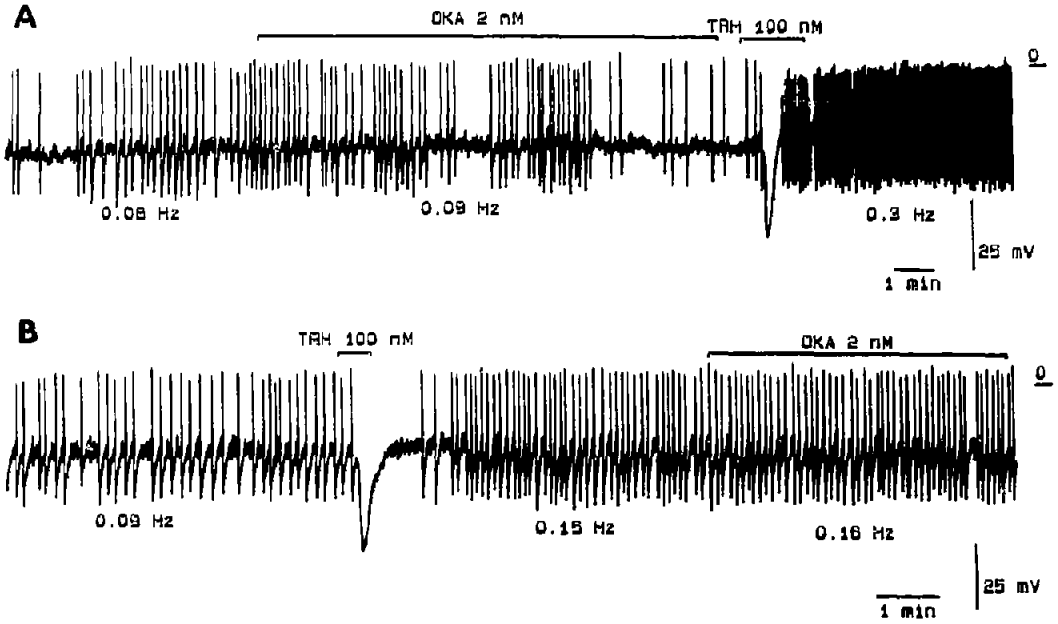

Fig. 2. Effect of 2 nM OKA on electrical activity in GH_3 cells. Application of 2 nM OKA to two different GH_3 cells either before (A) or after (B) 100 nM TRH is indicated by long horizontal bars. The frequency of APs under different conditions is shown below the current traces. 0 mV is represented by horizontal lines on the left.

ment of cells with TRH alone (see Table I). As shown in Fig. 3B, addition of 100 nM OKA once the second phase has been initiated, further increases the rate of production of APs up to a level equivalent to that reached after pretreatment with OKA (see also Table I).

Data obtained with different concentrations of OKA on the whole cell population studied are summarized in Table I. It is clear that maximal enhancement of TRH action is achieved when 2 nM OKA is used 5 min before perfusion with the hormone. However, only at a concentration of 100 nM is OKA able to produce further significant increases in electrical activity once the second phase of TRH action has started. It is important to emphasize that OKA, at the concentrations tested, does not modify the basal activity of the cells by itself. Thus, its effect is exclusively manifested as a potentiation of the TRH effect. Interestingly, three of the nine cells treated with 100 nM OKA showed increases of ca. 160% in the basal rate of spiking. Although not statistically significant when the whole cell population is considered, these data suggest that the basal level of phosphorylation may be quite different from cell to cell. It is also interesting to note that treatment of cells with either 2 or 100 nM OKA for 5 min does not significantly modify the magnitude of the initial hyperpolarization in response to a subsequent addition of 100 nM TRH. Thus, under these conditions, addition of hormone transiently increased the membrane potential from a resting value of -38.5 ± 0.7 mV ($n = 21$) to a maximum value of -69.1 ± 1.9 mV ($n = 21$). Furthermore, 33.6 ± 4.5 s ($n = 21$) were necessary for cells to recover from hyperpolarization.

The effects obtained after pretreatment of cells with 2 nM OKA suggest that PP-2A could be involved in regulation of delayed TRH actions. However, the requirement for 5 min of preincubation with inhibitor in order to obtain a measurable effect, may indicate that this time represents the period necessary to accumulate OKA to a level sufficient to produce a substantial inhibition of PP-1 (K_i ca. 10–500 nM; see above). Subsequently, we tested the effect of CL-A on the response to TRH. This compound, which inhibits PP-2A with a potency similar to that of OKA (IC_{50} 0.5–1 nM), also inhibits PP-1 at nearly identical concentrations in in vitro assays (IC_{50} about 2 nM; refs. [18–21]). In this case, a detectable effect of CL-A was only observed at concentrations above 10 nM. As in the case of 2 nM OKA, addition of 20 nM CL-A during the second phase of TRH action does not cause any further increase in the rate of firing (not shown). On the other hand, the basal level of firing is not modified by treatment of cells with 20 nM CL-A for 5 min ($112 \pm 12\%$ ($n = 10$) with respect to control without inhibitor). However, the addition of 100 nM TRH following this treatment with CL-A, elicited a second phase in which the frequency of firing was increased from a basal value of 0.24 ± 0.05 Hz up to 0.57 ± 0.05 Hz ($n = 10$). These data lend further support to the conclusion that a mechanism of phosphorylation–dephosphorylation is involved in the delayed effects of TRH. Furthermore, they suggest that

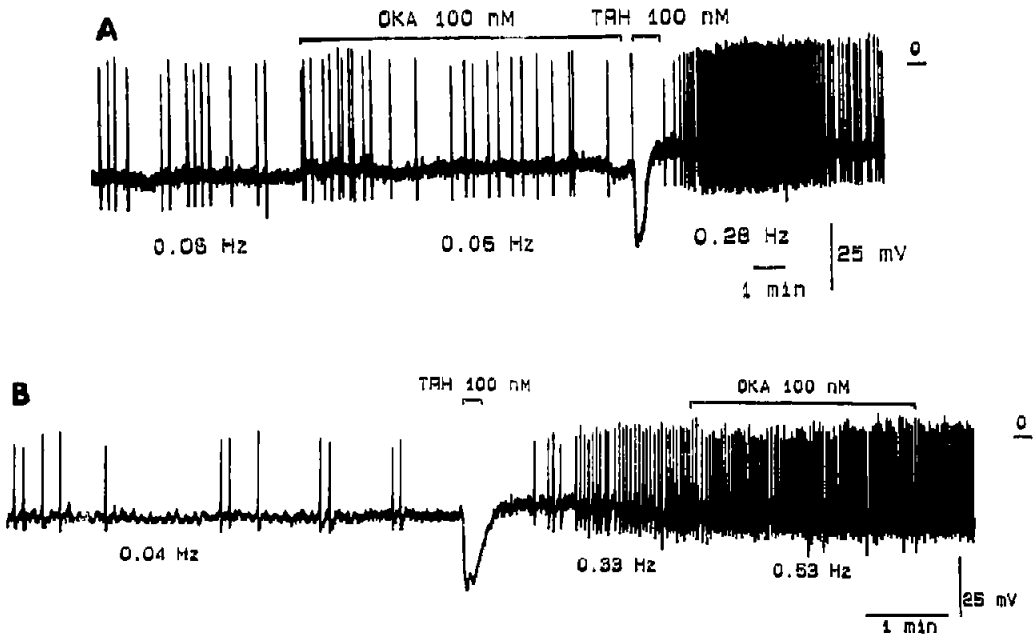

Fig. 3. Effect of 100 nM OKA on electrical activity in GH_3 cells. Application of 100 nM OKA to two different cells either before (A), or after (B), 100 nM TRH addition is indicated by long horizontal bars. The frequency of APs under different conditions is shown below the current traces. 0 mV is represented by horizontal lines.

PP-2A may be the enzyme involved in regulation of the electrical activity of GH_3 cells by TRH.

4. DISCUSSION

Our results demonstrate that the second phase of enhanced AP frequency induced by TRH in GH_3 cells is significantly potentiated by okadaic acid. This effect does not seem to be due to a non specific membrane effect of OKA since: (i) no effect of inhibitor was detected before treatment of cells with TRH; (ii) similar effects to those of OKA are observed with CL-A; (iii) the phase I response to TRH was not impaired by treatment of cells with the inhibitors; (iv) short incubation times and inhibitor concentrations as low as 2 nM are enough to elicit the effects of OKA; and (v) even at the highest concentration of OKA used in this study (100 nM), the enhancement of TRH effects is selectively exerted on inward rectifying K^+ currents (see [13]). It has been previously suggested that inhibition of an inwardly rectifying current by TRH constitutes an important factor regulating the resting potential and conductance underlying increased frequency of APs induced by the hormone in GH_3 cells [12,13]. Recent experiments from our laboratory indicated that treatment of patch-perforated GH_3 cells with OKA enhances the TRH-evoked inhibition of the inward rectifying current [13]. Since other K^+ currents present in these cells were not affected by this treatment, it was important to know whether the increase in frequency of firing is also potentiated by the phosphatase inhibitor. The use of perforated-patch conditions in order to correlate voltage clamp studies on the currents and current clamp analysis of firing frequency is especially relevant, since hormonal responses and membrane currents which are rapidly diminished using the whole-cell configuration of the patch-clamp technique, are fully preserved when perforated patches are used [5,6,11]. The parallel enhancement by OKA of TRH inhibition of the inward rectifying K^+ current [13] and increase of AP frequency (this report), indicate that this current plays a major role in determining the firing rate of GH_3 cells. It has been proposed that enhanced GH_3 cells excitability in response to TRH can be due to reductions in Ca^{2+}-activated K^+ currents secondary to inhibition of Ca^{2+} currents [24]. In fact, the TRH-induced inhibition of Ca^{2+}-dependent K^+ currents activated by depolarization has been previously demonstrated in GH_3 cells [5,11,24], and treatment of GH_4C_1 cells with OKA has been shown to greatly suppress the depolarization-activated currents [25]. However, those inhibitions do not seem to be the crucial regulator of the resting potential and conductance changes which cause the increased frequency of APs, since: (i) in GH_3 cells, Ca^{2+}-dependent K^+ currents [13] and Ca^{2+} currents studied with Cs^+-containing electrodes (Barros, F. and Delgado, L.M., unpublished results), remained unchanged in the presence of 100 nM OKA; and (ii) TRH elicited decreases in Ca^{2+}-dependent K^+ [13] and voltage-dependent Ca^{2+} currents [11,24], were not signifi-

cantly different in OKA-treated and -untreated cells ([13] and Barros, F., unpublished). Furthermore, these effects were readily reverted upon removal of the hormone also in OKA-treated cells. It has been previously shown that treatment of hepatocytes with OKA inhibits agonist induced stimulation of phospholipase C [26,27]. The fact that phase I of TRH induced hyperpolarization remains unchanged in the presence of OKA suggests that a different mechanism is involved in the effects of this inhibitor in GH_3 cells. It is also improbable that increases in voltage-dependent Ca^{2+} currents [23] are the cause of the observed increases in AP frequency, since Ca^{2+} currents of the T and/or L type were not affected by treatment with OKA in perforated-patch voltage-clamped GH_3 cells studied with Cs^+ containing electrodes (Delgado, L.M. and Barros, F., unpublished). Our results show that both OKA and CL-A are able to potentiate TRH increases in firing rate. This indicates that a phosphorylation–dephosphorylation mechanism is involved in the TRH effect. The fact that 20 nM CL-A is sufficient to reproduce the effects of 2 nM OKA may be due to certain differences in the ability of both drugs to permeate the cell membrane. However, preincubation of 5 min was necessary in both cases, even though the concentration of CL-A employed is far above that necessary to inhibit both PP-2A and PP-1. This suggests that this period of time is necessary to reach an intracellular concentration of the inhibitors sufficient to inhibit PP-2A, but not to reach a level at which PP-1 would be inhibited. Participation of TRH-induced Ca^{2+} release in activation of an OKA sensitive phosphatase of the calcineurin type does not seem to be involved in the effects reported here since both the increases in AP frequency and the reductions in inward rectifying currents [13] are produced in cells in which a Ca^{2+}-induced phase I of hyperpolarization cannot be detected. Clearly, knowledge of the PP implicated in the TRH effects must await further characterization of the enzymes present in GH_3 cells. Thus, it would be interesting to know whether a protein phosphatase of the PP-3 type recently described in bovine brain [28] is also present in GH_3 cells, and if both OKA and CL-A can inhibit the activity of this enzyme.

Acknowledgements: This work was supported by grants PB87-1046 and PB90-0789 from CICYT of Spain. We wish to thank Dr. A. Novelli for initial samples of okadaic acid and Dr. G.J. Kaczorowski for helpful comments on the manuscript.

REFERENCES

[1] Bjoro, T., Sand, O., Ostberg, B.C., Gordeladze, J.O., Torjesen, P., Gautvik, K.M. and Haug, E. (1990) Biosci. Reports 10, 189–199.
[2] Drummond, A.H., in: Inositol lipids in cell signalling (R.H. Michell, A.H. Drummond, M. Bushfield and C.H. MacPhee, Eds.), Academic Press, London, 1989, pp. 355–375.
[3] Gershengorn, M.C. (1986) Annu. Rev. Physiol. 48, 515–526.
[4] Ozawa, S. and Sand, O. (1986) Physiol. Rev. 66, 887–952.
[5] Barros, F., Delgado, L.M., Maciá, C. and de la Peña, P. (1991) FEBS Lett. 279, 33–37.
[6] Dufy, B., MacDermott, A. and Barker, J.L. (1986) Biochem. Biophys. Res. Commun. 137, 388–396.
[7] Barros, F., Katz, G.M., Kaczorowski, G.J., Vandlen, R.L. and Reuben, J.P. (1985) Proc. Natl. Acad. Sci. USA 82, 1108–1112.
[8] Dubinski, J.M. and Oxford, G.S. (1985) Proc. Natl. Acad. Sci. USA 82, 4282–4286.
[9] Oxford, G.S. and Wagoner, P.K. (1989) J. Physiol. 410, 587–612.
[10] Kramer, R.H., Kaczmarek, L.K. and Levitan, E.S. (1991) Neuron 6, 557–563.
[11] Simasko, S.M. (1991) Endocrinology 128, 2015–2026.
[12] Bauer, C.K., Meyerhof, W. and Schwarz, J.R. (1990) J. Physiol. 429, 169–189.
[13] Barros, F., Delgado, L.M., del Camino, D. and de la Peña, P. (1992) Pflügers Arch., in press.
[14] Gammon, C.M., Oxford, G.S., Allen, A.C., McCarthy, K.D. and Morell, P. (1989) Brain Res. 479, 217–224.
[15] Ostberg, B.C., Sand, O., Bjoro, T. and Haug, E. (1986) Acta Physiol. Scand. 126, 517–524.
[16] Haystead, T.A.J., Sim, A.T.R., Carling, D., Honnor, R.C., Tsukitani, Y., Cohen, P. and Hardie, D.G. (1989) Nature 337, 78–81.
[17] Cohen, P. and Cohen, P.T.W. (1989) J. Biol. Chem. 264, 21435–21438.
[18] Cohen, P., Holmes, F.B. and Tsukitani, Y. (1990) Trends Biochem. Sci. 15, 98–102.
[19] Bialojan, C. and Takai, A. (1988) Biochem. J. 256, 283–290.
[20] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[21] Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. and Hartshorne, D.J. (1989) Biochem. Biophys. Res. Commun. 159, 871–877.
[22] MacKintosh, C. and Klumpp, S. (1990) FEBS Lett. 277, 137–140.
[23] Hescheler, J., Mieskes, G., Rüegg, J.C., Takai, A. and Trautwein, W. (1988) Pflügers Arch. 412, 248–252.
[24] Kramer, R.H., Kaczmarek, L.K. and Levitan, E.S. (1991) Neuron 6, 557–563.
[25] White, R.E., Schonbrunn, A. and Armstrong, D.L. (1991) Nature 351, 570–573.
[26] Garcia-Sainz, J.A., Macias-Silva, M. and Romero-Avila, M.T. (1991) Biochem. Biophys. Res. Commun. 179, 852–858.
[27] Mattingly, R.R. and Garrison, J.C. (1992) FEBS Lett. 296, 225–230.
[28] Honkanen, R.E., Zwiller, J., Daily, S.L., Khatra, B.S., Dukelow, M. and Boynton, A.L. (1991) J. Biol. Chem. 266, 6614–6619.