Research Paper

Roles of Protein Kinase A (PKA) and PKC on Corticotropin-Releasing Hormone (CRH)-Induced Elevation of Cytosolic Calcium from Extra- and Intra-cellular Sources

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ABSTRACT

Corticotropin-releasing hormone (CRH) affects cytosolic calcium ion levels. The aim of the present work was to examine the role of protein kinase A (PKA)- and PKC-dependent signalling pathways in mediating the effect of CRH on calcium ion influx (from extra-cellular sources) and calcium ion mobilization (from intra-cellular stores). In this study, we employed a well-known model of neural crest-derived cells, the PC12 rat pheochromocytoma cell line. We found that CRH increased the concentration of cytosolic calcium ions in calcium-rich and in calcium-free media. In both conditions, an inhibitor of PKA phosphorylation abolished the effect of CRH. In contrast, the inhibitor of PKC phosphorylation blocked the effect of CRH only in calcium-free conditions. The phorbol ester PMA, activator of PKC, accelerated the steep of the curve of cytosolic calcium ion increase from intra-cellular stores. These data suggest that: (a) CRH induces calcium ion entrance into the cytoplasm from both extra-cellular sources (influx) and from intra-cellular stores (mobilization); (b) the PKA-dependent signalling pathway mediates both effects of CRH; and (c) the PKC-dependent signalling pathway mediates only the CRH-induced mobilization of calcium ions from intra-cellular stores. Thus, this is the first report demonstrating that distinct signalling pathways control the effects of CRH on calcium ion influx and on calcium ion mobilization from intra-cellular stores.

Key words: Calcium signalling, Corticotropin-Releasing Hormone, PKA, PKC, PC12 Pheochromocytoma

ABBREVIATIONS

CRH	corticotropin-releasing hormone
PKC	protein kinase C
PKA	protein kinase A
PMA	phorbol 12-myristate 13-acetate
CRHR1	CRH type 1 receptor

INTRODUCTION

The fluctuations of cytosolic calcium ion levels represent part of signalling pathways through which extracellular signals affect cellular functions including exocytosis, gene expression, contraction, and programmed cell death (apoptosis). Cytosolic calcium ions derive from two sources: (a) from influx of extra-cellular calcium, either via voltage-gated or ligand-dependent channels, and (b) from the mobilization of calcium from intra-cellular stores, the most significant of which is the endoplasmic reticulum.

It is now well established that calcium ion signalling pathways mediate a significant number of the biological effects of corticotropin releasing hormone (CRH) and these effects are possibly of particular importance with regard to cell function. It was initially shown that CRH induces calcium ion influx in AtT-20 corticotroph cells possibly via the PKC signalling pathway[1]. It was later shown that the effect of CRH on ACTH secretion is largely dependent on calcium ion influx through activation of voltage-gated L-type Ca ion channels, an effect accompanied by a simultaneous CRH-mediated induction of the expression of these channels[2]. Similarly, the relaxing activity of CRH on ileal smooth muscle cells appears to involve mobilization of calcium ions from intracellular stores within the sarcoplasmic reticulum[3]. This effect of CRH appears to be mediated also by a PKA-dependent signalling pathway. Furthermore, CRH inhibits receptor-mediated intracellular calcium responses in a locus coeruleus-like cell line, the CATH.a, an effect involving PKA[4]. In addition, CRH induces cell shape changes in invertebrate immunocytes via both the PKA and PKC signalling pathways[5,6] while sauvagine [a CRH type 1 receptor (CRHR1) and CRHR2 receptor agonist] induces the mobilization of calcium ions from intracellular stores[7]. Finally, the inhibitory effect of CRH on the proliferation of mouse melanoma cells is mediated by the CRHR1 receptor and involves calcium ion signalling[8].

Classically, CRH stimulates the expression as well as the secretion of ACTH in rat anterior pituitary corticotrophs and in the murine AtT-20 cell line via the Gs - adenyl cyclase - cAMP – PKA signalling pathway. However, it has been established that CRH also affects the Gq - phospholipase C-inositol 1,4,5-tris-phosphate - PKC pathway in pituitary, Leydig cells, adrenals, placenta, immunocytes, myometrium, and in the hippocampus[5,9-11]. Furthermore, it has been shown that in human epidermoid cells, CRH induces the activity as well as the translocation of PKC isoenzymes[12]. Finally, exposure of neuroblastoma cells to phorbol esters, activators of PKC, results in up-regulation of CRH binding sites suggesting an additional mechanism through which the PKC signalling pathway may modulate some of the biological effects of CRH[13].

Based on the data mentioned above, it has been hypothesized that the effect of CRH on cytosolic calcium ion levels may be mediated by either the PKA or the PKC signalling pathways. It should be noted that a recent report states that this may be true for the skin[14]. Indeed, in corticotroph cells, although PKA mediated the stimulatory effect of dBcAMP and forskolin on calcium ion influx (through voltage-gated Ca ion channels), it only partially mediated the effect of CRH, giving the impression that the effect of CRH on calcium ion influx may be also mediated by a cAMP-independent mechanism[5,15]. Thus, it has been proposed that CRH stimulates calcium ion influx, mainly via L-type Ca ion channels. The observed calcium ion influx, which is independent of action potentials (membrane depolarization) and mediated by PKC, may involve P-type calcium ion channels[16].

The aim of the present work was to elucidate the role of the PKA and PKC signalling pathways in the mediation of the stimulatory effect of CRH on cytosolic ion levels from extra-cellular and intra-cellular calcium ion sources. In this study, we employed a well-known model of neural crest-derived cells, the PC12 rat pheochromocytoma cell line. Our data advance a novel hypothesis regarding the effect of CRH on calcium ion trafficking.

MATERIALS AND METHODS

Reagents and Antibodies

Rat/human recombinant CRH was purchased from Sigma Chemicals Co. (St. Louis, MO). The inhibitor of PKC phosphorylation, the pseudo-substrate myristoylated alanine-rich PKC[20-28] (Myr-N-FARK-GALRQ-NH$_2$) and the inhibitor of PKA phosphorylation, myristoylated PKA[14-22] (Myr-GRTGR-RNAI-NH2) were obtained from Calbiochem (La Jolla, CA) while the activator of conventional and novel PKC isoenzymes, phorbol 12-myristate 13-ace-

tate (PMA), was purchased from Sigma. The inhibitor of the conventional PKC isoenzymes PKCα/β is a pseudosubstrate sequence from PKCα and PKCβ that is N-terminal myristoylated to allow membrane permeability. The inhibitor of PKCα/β specifically inhibits TPA activation of MARCKS phosphorylation in fibroblast primary cultures (IC50= 8 μM). The inhibitor exhibits 98% inhibition at 100 μM. RPMI Medium 1640, L-glutamine, HEPES, penicillin/ streptomycin, horse serum and fetal calf serum (FCS) were purchased from Gibco (Gibco-BRL Co, MD), while bovine serum albumin (BSA) was obtained from Sigma. All sterile tissue apparatus were obtained from Corning (NY, NY). All other chemicals and reagents were obtained from Sigma, unless stated otherwise.

The PC12 rat pheochromocytoma cell line

PC12 cells were obtained from two sources: Dr. M. Greenberg (Children's Hospital, Boston, MA, U.S.A.), and the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI Medium 1640 containing 10 mM L-glutamine, 15 mM HEPES, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10% horse serum and 5% FCS at 5% CO_2 and 37 ˚C. One day before the experiment, the initial culture media were changed with serum-free media supplemented with 0.1% BSA.

Measurement of cytosolic calcium ions

PC12 were detached from culture flasks by vigorous shaking, washed and resuspended in Ca^{2+} medium (140mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 10mM Hepes, 5mM D-glucose) at a cell density of 0.5 million per ml. Subsequently, they were incubated for 30min at 25˚C in the dark with 5μM Fura 2/ AM (Molecular Probes, Leiden, the Netherlands), centrifuged (1500xg, 10 min), resuspended in either calcium rich or calcium free media (without calcium ions plus 1mM EGTA, final concentration of calcium ions is less than 0.1nM), and transferred to quartz cuvettes. Fluorescence was measured at time intervals of 10 sec at two excitation wavelengths (340nm and 380nm) and a single emission wavelength (510 nm) with a Perkin Elmer LS-3B Fluorescence Spectrometer. Cytosolic calcium ion responses were expressed as the ratio of peak fluorescence intensities (measured at 510 nm) produced by Fura 2 using excitation wavelengths of 340 and 380 nm. In its free form, Fura 2 has high excitation efficiency at 380 nm and

low excitation efficiency at 340 nm. With calcium bound, the reverse is true: at 380 nm Fura 2 fluoresces with low excitant efficiency and at 340 nm with high excitant efficiency. This ratio (340 nm over 380 nm fluorescence intensities) is an index of calcium concentration. Therefore, changes in calcium concentration are expressed as change in ratio units (DR). The fluorescence ratios are directly proportional to the absolute calcium concentrations.

RESULTS

Effect of CRH ± PKA or PKC inhibitors on calcium ion influx

The effect of CRH on calcium ion influx from extra-cellular sources was examined in the first set of experiments in which PC12 cells were incubated in calcium ion rich media and subsequently exposed to CRH. The measurement of cytosolic calcium ion levels started within seconds following the application of CRH and continued for 1500 sec. Figure 1, panel A depicts the effect of CRH at 1 nM which induced an acute elevation of cytosolic calcium ions. Figure 1, panel D depicts the effect of the simultaneous presence of the PKA phosphorylation inhibitor myristoylated PKA[14-22] at 100 μM on CRH-induced calcium ion elevation in PC12 cells in a calcium rich environment. Indeed, the PKA inhibitor prevented the stimulatory effect of CRH suggesting that PKA-dependent signalling pathways mediate its effect. Compared to CRH alone, the simultaneous presence of the activator of PKC phorbol 12-myristate 13-acetate (PMA) at 200 nM (Figure 1, panel B) or of the PKC phosphorylation inhibitor pseudo-substrate myristoylated alanine-rich PKC[20-28] at 100 μM (Figure 1, panel E) did not significantly alter the steep of the curve depicting the rate of cytosolic calcium ion elevation suggesting that the PKC signalling pathway was not part of the CRH-induced calcium ion influx.

Effect of CRH ± PKA or PKC inhibitors on the mobilization of calcium ions from intracellular stores

The effect of CRH on the mobilization of calcium ion from intra-cellular sources was examined in the first set of experiments in which PC12 cells were cultured in calcium ion free media and subsequently exposed to CRH. Figure 2, panel A depicts the effect of CRH at 1 nM which induced an elevation of cytosolic
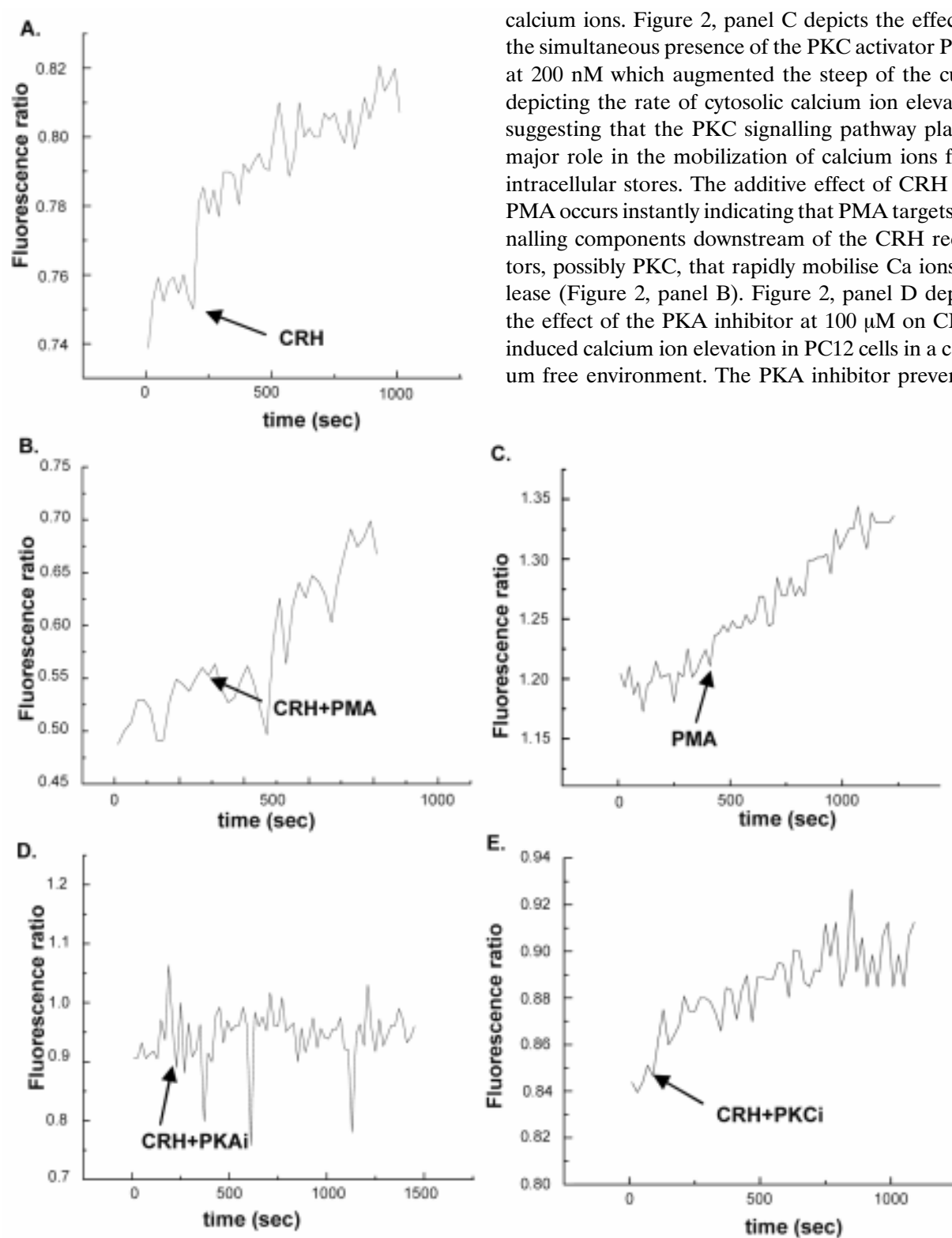
calcium ions. Figure 2, panel C depicts the effect of the simultaneous presence of the PKC activator PMA at 200 nM which augmented the steep of the curve depicting the rate of cytosolic calcium ion elevation suggesting that the PKC signalling pathway plays a major role in the mobilization of calcium ions from intracellular stores. The additive effect of CRH and PMA occurs instantly indicating that PMA targets signalling components downstream of the CRH receptors, possibly PKC, that rapidly mobilise Ca ions release (Figure 2, panel B). Figure 2, panel D depicts the effect of the PKA inhibitor at 100 μM on CRH-induced calcium ion elevation in PC12 cells in a calcium free environment. The PKA inhibitor prevented

Figure 1. Effect of CRH on calcium ion influx from extra-cellular sources. Panel A depicts the effect of CRH at 1 nM. Panel B depicts the effect of CRH + PMA at 200 nM. Panel C depicts the effect of PMA at 200 nM. Panel D depicts the effect of CRH + the PKA inhibitor at 100 μM. Panel E depicts the effect of CRH + PKC inhibitor at 100 μM. Cytosolic calcium ion levels were measured for 1500 sec. The arrows indicate the time point at which the stimulus was applied on the cells.

completely the stimulatory effect of CRH suggesting that PKA-dependent signalling pathways mediate the effect of CRH. Figure 2, panel E depicts the effect of the PKC inhibitor at 100 μM on CRH-induced elevation of cytosolic CRH from intra-cellular stores i.e. in a calcium free environment. The PKC inhibitor blocked completely the effect of CRH suggesting that the PKC signalling pathway mediates the effect of CRH on calcium ion mobilization from intra-cellular sources.

Taken together, the above mentioned two sets of experiments on the effect of CRH on calcium rich (Figure 1) or calcium free (Figure 2) environments indicate that while the PKA signalling pathway mediates the stimulatory effect of CRH on both calcium ion influx and calcium ion mobilization from intra-cellular sources, the PKC signalling pathway mediates only the latter.
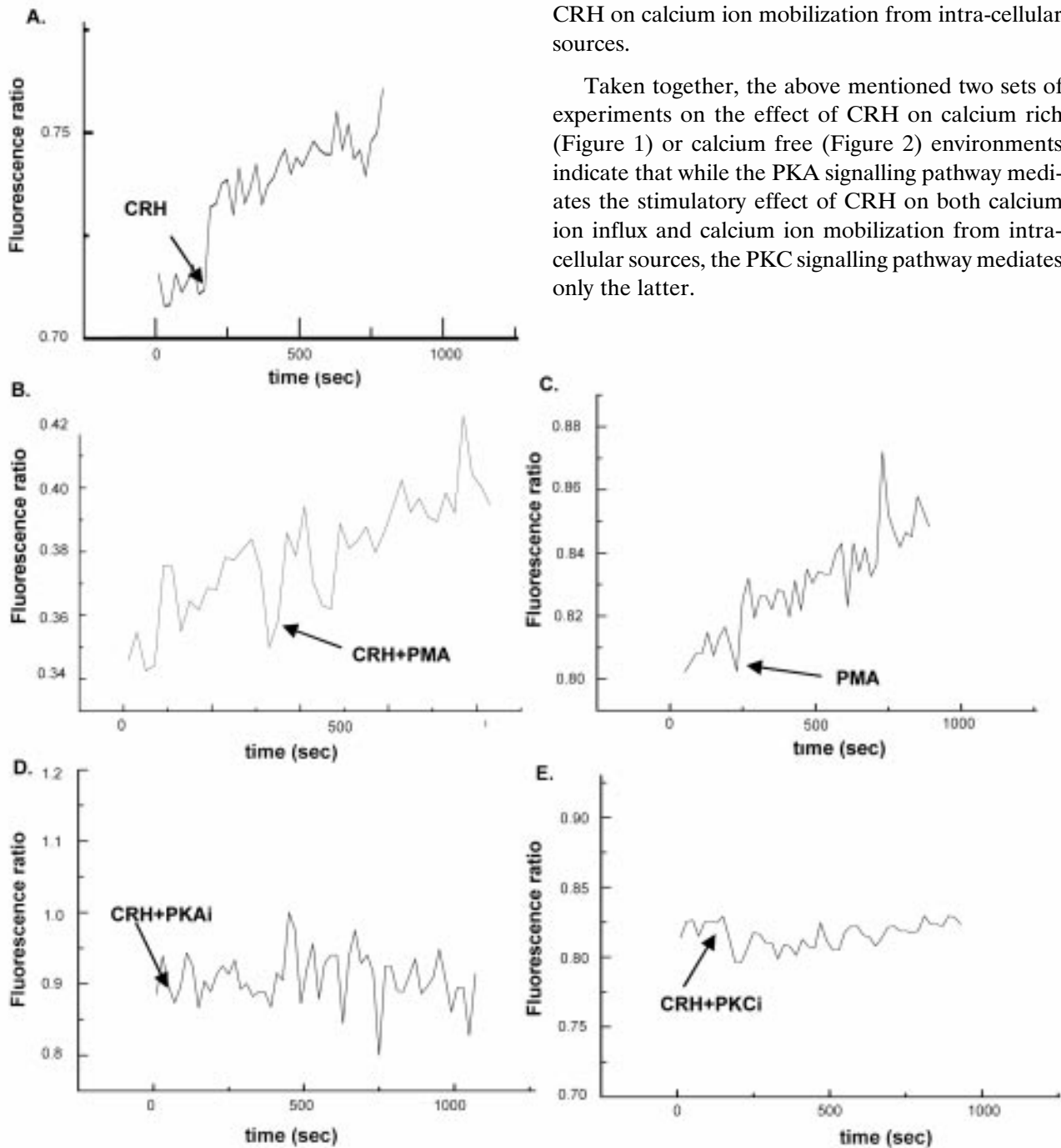

Figure 2. Effect of CRH on calcium ion influx from intra-cellular sources. Panel A depicts the effect of CRH at 1 nM. Panel B depicts the effect of CRH + PMA at 200 nM. Panel C depicts the effect of PMA at 200 nM. Panel D depicts the effect of CRH + the PKA inhibitor at 100 μM. Panel E depicts the effect of CRH + PKC inhibitor at 100 μM. Cytosolic calcium ion levels were measured for 1500 sec. The arrows indicate the time point at which the stimulus was applied on the cells.

DISCUSSION

We found that CRH induces the entrance of calcium ions into the cytoplasm from (a) extra-cellular sources (via induction of its cellular influx) and (b) intra-cellular sources (via calcium ion mobilization from intra-cellular sources the most important of which is the endoplasmic reticulum). To this point, our data are in agreement with published reports showing that CRH induces calcium ion entrance from extra-cellular[2,5,15-18] and/or intra-cellular sources[3,6,7,15,16,19,20].

We have further detected that PKA and PKC signalling pathways play distinct roles in CRH-induced elevation of cytosolic calcium ions. Indeed, our data suggest that the PKA-dependent pathway mediates both effects of CRH i.e. the induction of calcium ion influx as well as its mobilization from intracellular stores while the PKC-dependent pathway appears to be associated only with the mobilization of calcium ions from intracellular stores. It should be noted here that classically, the adenyl cyclase - cAMP - PKA signalling pathway was associated with the influx of extra-cellular calcium ions (either via voltage-gated or ligand-dependent channels) while the phospholipase C (PLC) – PIP2 – IP3 – IP3 receptor (located on the endoplasmic reticulum) was associated with the mobilization of calcium ions from intracellular stores[21]. The exact role and significance of the PLC - PIP2 - DAG – PKC pathway is uncertain. During the last few years it has been shown that CRH affects calcium ion trafficking mainly via the PKA-dependent signalling pathway[4,15,22], although the PKC signalling pathway was also associated with calcium ion influx from extra-cellular sources, possibly via a P-type calcium ion channel[5,16]. Our findings clearly demonstrate for the first time that the effect of CRH on extra-cellular calcium ion influx is mediated exclusively by the PKA signalling pathway. Indeed, the PKC pathway is not involved in CRH-induced extra-cellular calcium ion influx. In addition, we found that the effect of CRH on calcium ion mobilization from intra-cellular sources appears to be mediated by both the PKA and the PKC signalling pathways.

We previously published our findings showing that CRH induces PC12 cell apoptosis via the PKC signalling pathway[23]. We now report that CRH mobilizes cytosolic calcium ions from intracellular sources via the same signalling pathway. It has been shown that calcium ion mobilization from the endoplasmic reticulum activates cell death cascades via calcium ion influx into the mitochondria, which releases cytochrome c, and by calcium ion influx into the nucleus, where it participates in the apoptotic process. This is accomplished by induction of apoptotic gene transcription, caspase activation, and nuclear DNA fragmentation[24]. Indeed, the calcium-mediated pathways appear to be extremely important in neuronal cell apoptosis[25]. Thus, our data on CRH-induced mobilization of intracellular calcium ions via PKC and its effect on apoptosis via the same signalling pathway strengthens the hypothesis that CRH may be associated with neuro-degenerative diseases.

ACKNOWLEDGMENT

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