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Corticotropin-releasing factor-like peptides increase cytosolic [Ca^{2+}] in human epidermoid A-431 cells

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Abstract

This study investigated whether sauvagine and urotensin I change $[Ca^{2+}]_i$ in human epidermoid A-431 cells and whether these changes are correlated with their anti-edema properties in vivo. A-431 cells were used because they possess the corticotropin-releasing factor (CRF) receptor 2. Treatment with either sauvagine or urotensin I led to an immediate increase in $[Ca^{2+}]_i$, the magnitude of which depended on the concentration of the drug. Sauvagine was more effective than urotensin I, with a median effective concentration (EC_{50}) of 1.4 ± 0.2 fM, compared to an EC_{50} of 66 ± 7 fM for urotensin I. Both were more effective at stimulating increases in $[Ca^{2+}]_i$ than CRF (EC_{50} of 6.8 ± 0.1 pM). There was a correlation between the EC_{50} for increasing $[Ca^{2+}]_i$ and the median effective dose (ED_{50}) for inhibiting edema induced by heating rat paw ($r = 0.99$). Removal of extracellular Ca^{2+} or incubation with La^{3+} eliminated the increase in $[Ca^{2+}]_i$ stimulated by either peptide. Pretreatment with a CRF receptor antagonist reduced the increase in $[Ca^{2+}]_i$ by these peptides. This occurred in an antagonist concentration-dependent manner, with median inhibitory concentrations (IC_{50}) of 1.99 ± 0.04 nM and 0.85 ± 0.04 nM, respectively. Both pertussis toxin (an inhibitor of G proteins) and U-73122 (an inhibitor for inositol trisphosphate ($InsP_3$) production) partially inhibited the increases. $InsP_3$ was measured to determine whether these peptides mobilized Ca^{2+} from an $InsP_3$-sensitive store. Both sauvagine and urotensin I increased $InsP_3$. The $InsP_3$ increases were inhibited by U-73122 and CRF antagonist, but not by removal of external Ca^{2+}. Both peptides elevated protein tyrosine phosphorylation. In summary, these peptides increase $[Ca^{2+}]_i$ as a result of Ca^{2+} influx via CRF receptor-operated Ca^{2+} channels coupled to pertussis toxin-sensitive G proteins and a Ca^{2+} mobilization from $InsP_3$-sensitive Ca^{2+} pools. Their in vivo effectiveness at inhibiting edema is related to their respective capacities to stimulate elevations of $[Ca^{2+}]_i$, supporting a role for intracellular Ca^{2+} in this process. © 1997 Elsevier Science B.V.

Keywords: Sauvagine; Urotensin I; Corticoliberin; CRF (corticotropin-releasing factor); Epithelium; Edema; Ca^{2+}; Inositol trisphosphate; CRF receptor; Skin; Tyrosine; Phosphorylation

1. Introduction

Sauvagine (present in frog skin), urotensin I (present in sucker fish tail) and corticotropin releasing factor (CRF) are members of the corticoliberin superfamily. Sauvagine and urotensin I consist of 40 and 41 amino-acid residues, respectively, with 45–55% homology to human/rat CRF. In isolated rat corticotrophs, sauvagine and urotensin I stimulate the release of adrenocorticotropin with potencies equivalent to ovine CRF (Rivier et al., 1983). However, these peptides are 5–10 times more potent than CRF in dilating the mesenteric arterial beds of the rat and dog (Lederis et al., 1985; MacCannell et al., 1982). Activities of the peptides analogous to CRF were found in studies to inhibit edema formation and protein extravasation caused by heat exposure and antidromic stimulation of the saphenous nerve. Sauvagine and urotensin I were 13.4 and 4 times more potent than CRF in inhibiting edema formation and protein extravasation (Wei and Kiang, 1989).

The mechanisms underlying the inhibitory capacities of sauvagine and urotensin I are not understood. CRF activity has been shown to involve Ca^{2+}, because removal of external Ca^{2+}, blockage of Ca^{2+} entry, depletion of intracellular Ca^{2+} pools, or treatment with calmodulin antagonists inhibit CRF (Boutillier et al., 1991; Lee and Lin, 1991; Won and Orth, 1990). These findings suggest that Ca^{2+} acts as a trigger for CRF activity.

CRF has been shown to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in human adrenocorticotropin

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(ACTH)-secreting pituitary adenoma cells and normal rat small ovoid corticotrophs (Guerineau et al., 1991) and in human epidermoid A-431 cells (Kiang, 1994). The increase in $[Ca^{2+}]_i$ induced by CRF is thought to be mediated by CRF receptors coupling to pertussis toxin-sensitive G proteins. Whether the mechanisms of action of sauvagine and urotensin I are similar to CRF is not known.

The physiological significance of changes in $[Ca^{2+}]_i$ has been extensively documented. It is likely that an increase in $[Ca^{2+}]_i$ serves as a common signal to trigger a variety of cellular functions. For example, in mouse pituitary AtT-20 cells, the increased transcription of c-*fos* mRNA induced by CRF depends on Ca^{2+} (Boutillier et al., 1991). ACTH release in dispersed rat anterior pituitary cells is Ca^{2+}-dependent (Won and Orth, 1990), and the heat stress-induced heat shock protein 72 kDa in human epidermoid A-431 cells is induced by Ca^{2+} ionophores (Ding et al., 1996; Kiang, 1994). It is likely that the anti-edema property of CRF is mediated by the increase in $[Ca^{2+}]_i$ induced by CRF.

In this study we have characterized the effect of sauvagine and urotensin I on $[Ca^{2+}]_i$. Both peptides increased $[Ca^{2+}]_i$, which occurred as a result of both the receptor-mediated entry and mobilization of Ca^{2+} from $InsP_3$-sensitive Ca^{2+} pools. Sauvagine and urotensin I also independently increased $InsP_3$, perhaps mediated by activation of protein tyrosine kinases.

2. Materials and methods

2.1. Cell culture

Human epidermoid carcinoma A-431 cells (American Type Culture Collection, Rockville, MD, USA) were grown on glass cover slips (9 × 35 mm, Clay Adams, Lincoln Park, NJ, USA) incubated at 37°C in an atmosphere of 5% CO_2. The tissue culture medium was Dulbecco's modified Eagle medium supplemented with 0.03% glutamine, 4.5 g/l glucose, 25 mM HEPES, 10% fetal bovine serum, 50 μg/ml penicillin and 50 U/ml streptomycin (Gibco BRL, Gaithersburg, MD, USA). Cells were fed every 3–4 days. Cells from passage 28–45 were used for experiments.

2.2. Intracellular Ca^{2+} measurements

Confluent monolayers of cells grown on glass cover slips (5×10^5 cells/slip) were loaded with 5 μM fura-2 acetoxymethyl ester (fura-2AM) plus 0.2% pluronic F-127 (to make cells more permeable to the probe) at 37°C for 60 min. Cells were washed with Na^+ Hanks' buffer before fluorescence measurements. The method to determine $[Ca^{2+}]_i$ has been described previously (Grynkiewicz et al., 1985; Kiang, 1991). Briefly, the confluent monolayer of cells was placed in a thermostatically controlled cuvette that was kept at a constant temperature of 37°C. The fluorescence signal from 5×10^5 cells/slip was measured with a PTI DeltaScan spectrofluorometer (Photon Technology International, South Brunswick, NJ, USA) with emission at 510 nm and dual excitation at 340 and 380 nm (slit width 4 nm). Autofluorescence from cells not loaded with the dye was in the range of 3000–4000 photons/s and was subtracted from the fura-2 signal. Fura-2 leaked out of A-431 cells at a rate of $0.38 \pm 0.01\%$/min ($n = 3$) at 37°C. To minimize any contribution to the fluorescence signal resulting from dye in the medium, cells were washed thoroughly in Hanks' solution before measurement of $[Ca^{2+}]$. $[Ca^{2+}]_i$ was determined according to the method of Grynkiewicz et al. (1985). For experiments performed in the absence of extracellular Ca^{2+}, cells were incubated in the Ca^{2+}-free buffer containing 10 mM EGTA for 1 min prior to treatments with drugs.

2.3. Inositol trisphosphates measurements

Inositol trisphosphates ($InsP_3$), including Ins(1,3,4)P_3 and Ins(1,4,5)P_3, were measured as described by Berridge (1983) and this laboratory (Kiang and McClain, 1993). In brief, cells were grown in 6-well culture plates and incubated with *myo*-[^{3}H]inositol (2 μCi/ml, 0.22 nmol/ml, Dupont/NEN, Boston, MA, USA) for 24 h. Cells were washed twice with Na^+ Hanks' solution, then incubated with CRF for 5 s, 1, 5, or 10 min. The reaction was stopped by adding 3 ml ice-cold 4.5% $HClO_4$/Na^+ Hanks' solution (2:1, v/v). Each plate was placed on ice for 30 min and the cells were removed by scraping. Cells were pelleted by centrifugation ($750 \times g$) and the supernatants were collected. The pH of the supernatants was adjusted to pH 8.0 with KOH, and the samples were stored at −70°C until analysis. An aliquot (100 μl) of supernatant, which contained all of the [^{3}H]inositol and [^{3}H]polyphosphoinositol metabolites, was counted to determine total radioactivity in the cells. [^{3}H]$InsP_3$ was eluted from a Dowex AG 1-x8 resin column with 100 mM formic acid in 1.0 M ammonium formate (38 ml). Radioactivity was determined by mixing 1 ml of the eluent with 10 ml of Aquasol scintillation cocktail (Dupont/NEN) and counted with a scintillation counter. The amount of $InsP_3$ was then expressed as a percentage of total cpm in each well.

2.4. Western blots

Monolayers of cells (5×10^6) were removed from cover slips by trypsinization and resuspended in Na^+ Hanks' solution containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). The suspension was sonicated and the protein determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, South Richmond, CA, USA). Each sample (50 μg protein) was loaded onto precasted 10% sodium dodecyl sulfate-polyacrylamide gels (Novex, San Diego, CA, USA). After electrophoresis, the separate proteins were transferred to a nitrocellulose mem-

brane (MSI Micron Separations, Westborough, MA, USA) using a Novex blotting module. Phosphotyrosine was detected using an anti-phosphotyrosine antibody and the manufacturer's protocol.

2.5. Solutions

Na^+ Hanks' buffer contained 145 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.6 mM $CaCl_2$ and 10 mM HEPES (pH 7.4 at 24°C). In Ca^{2+}-free Hanks' buffer, $CaCl_2$ was replaced with 10 mM EGTA and pH was adjusted to 7.4 at 24°C.

2.6. Statistical analysis

All data are expressed as mean ± S.E.M. Analysis of variance, studentized range test and Student's *t*-test were used for comparison of groups with $P < 0.05$ as the significant level (Sokal and Rohlf, 1969). Curve fitting was performed and median effective concentrations (EC_{50}) and median inhibitory concentrations (IC_{50}) were calculated using the Inplot program (GraphPad, San Diego, CA, USA).

2.7. Chemicals

CRF, sauvagine and α-helical CRF-(9–41) were purchased from Peninsula Laboratories (Belmont, CA, USA) and urotensin I was obtained from Bachem (Torrance, CA, USA). Other chemicals used in this study were fura-2/AM, pluronic acid F-127 (Molecular Probes, Eugene, OR, USA), pertussis toxin (Sigma, St. Louis, MO, USA) and horseradish peroxidase-conjugated anti-phosphotyrosine (Upstate Biotechnology, Lake placid, NY, USA). 1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-1*H*-tyrrole-2,5-dione (U-73122) and 1-{6-[(17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino]hexyl}-2,5-pyrrolidine-dione (U-73343) were provided by Upjohn (Kalamazoo, MI, USA).

3. Results

3.1. Effects of sauvagine and urotensin I on $[Ca^{2+}]_i$

The resting $[Ca^{2+}]_i$ in adherent cells in the presence of external Ca^{2+} often varied from one experiment to the next. It is believed that this variation was associated with variations in status of the cells and the conditions of measurements. Therefore, each experiment was conducted with a control, and comparisons were made to that control only. However, all of the control values from these experiments were combined to calculate a mean baseline $[Ca^{2+}]_i$ of 100 ± 8 nM ($n = 82$).

When cells were treated with sauvagine or urotensin I, $[Ca^{2+}]_i$ increased in a drug concentration-dependent manner. $[Ca^{2+}]_i$ increased immediately and remained elevated up to 5 min. The tracing was not routinely followed longer than 5 min. The EC_{50} values for sauvagine and urotensin I were 1.4 ± 0.2 fM and 66 ± 7 fM, respectively (Fig. 1). Both were more effective at increasing $[Ca^{2+}]_i$ than CRF, which has an EC_{50} of 6.8 ± 0.1 pM.

For all subsequent experiments, the peptides were used at concentrations above those required to stimulate maximal increases in $[Ca^{2+}]_i$. Those concentrations were 42 pM CRF, 1 pM sauvagine and 10 pM urotensin I. Although these concentrations represent various multiples of their respective EC_{50} values, we found that increases in the concentrations of any of the peptides above the levels required to maximally stimulate $[Ca^{2+}]_i$ did not have any additional effects on the cells (Fig. 1).

The relative potencies of the peptides in these studies were the same as those observed to inhibit edema and protein extravasation caused by heating in in vivo studies. Previously this laboratory (Wei and Kiang, 1989) reported that ED_{50} of these peptides for inhibiting edema formed on rat paw caused by heat was 0.44, 1.5 and 5.9 nmol/kg, respectively. The correlation between the EC_{50} for increasing $[Ca^{2+}]_i$ in A-431 cells and the ED_{50} for inhibiting edema in rats is shown in Fig. 2. The correlation coefficient of 0.99 strongly suggests that increases in $[Ca^{2+}]_i$ stimulated by these peptides are related to their anti-edema properties.

3.2. Effects of Ca^{2+} channel blockers on increases in $[Ca^{2+}]_i$

The increase in $[Ca^{2+}]_i$ induced by sauvagine or urotensin I did not occur when cells were incubated in Ca^{2+}-free buffer containing 10 mM EGTA (71 ± 8 nM, $n = 10$); resting $[Ca^{2+}]_i$ decreased instead. In the presence of 10 μM La^{3+}, sauvagine and urotensin I also failed to increase $[Ca^{2+}]_i$ (Table 1). These observations suggest that the increases in $[Ca^{2+}]_i$ stimulated by sauvagine or

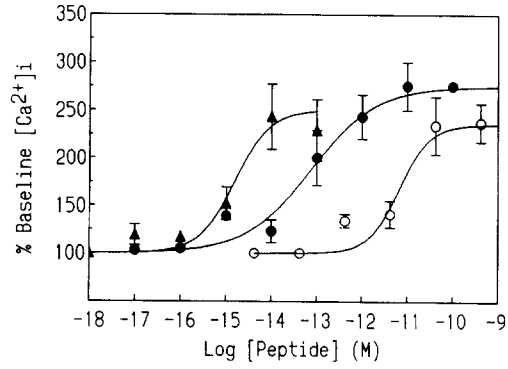

Fig. 1. Sauvagine and urotensin I increase $[Ca^{2+}]_i$. Human epidermoid A-431 cells were treated with various concentrations of peptide immediately prior to determination of $[Ca^{2+}]_i$. EC_{50} values for sauvagine and urotensin I are 6.8 ± 0.1 pM, 1.4 ± 0.2 fM, and 66 ± 7 fM, respectively ($n = 3$–6). (○) CRF; (▲) sauvagine; (●) urotensin I.

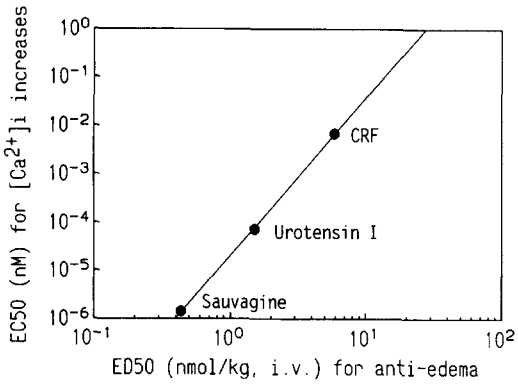

Fig. 2. $[Ca^{2+}]_i$ EC_{50} values for h/r-CRF, sauvagine, and urotensin I are positively correlated with ED_{50} values for anti-edema activity. The respective $[Ca^{2+}]_i$ EC_{50} values for CRF, sauvagine and urotensin I are 6.8 ± 0.1 pM, 1.4 ± 0.2 fM and 66 ± 7 fM. The ED_{50} value of these peptides for inhibiting edema in rats was 0.44, 1.5 and 5.9 nmol/kg, respectively. Correlation coefficient = 0.99.

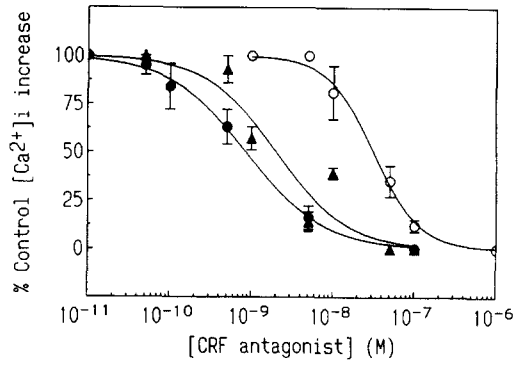

Fig. 3. CRF antagonist inhibits increases in $[Ca^{2+}]_i$ induced by sauvagine and urotensin I. Cells were treated with different concentrations of CRF antagonist for 1 min before a challenge with 42 pM CRF, 1 pM sauvagine, or 10 pM urotensin I. IC_{50} values for CRF, sauvagine, and urotensin I are 33 ± 2 nM, 1.99 ± 0.04 nM and 0.85 ± 0.04 nM, respectively ($n = 3$–6). (○) CRF; (▲) sauvagine; (●) urotensin I.

urotensin I are due to Ca^{2+} entry from the external buffer through Ca^{2+} channels. To determine whether these channels are voltage-gated, cells were treated with 10 μM verapamil for 1 min before challenge with each peptide. Verapamil did not inhibit either sauvagine- or urotensin I-induced increases in $[Ca^{2+}]_i$ (Table 1).

3.3. Effect of receptor antagonist on increases in $[Ca^{2+}]_i$

CRF receptors of two types have been shown to be present in a variety of cells and organs (Chen et al., 1993; Lovenberg et al., 1995a,b; Perrin et al., 1993). Our preliminary data indicate that human A-431 cells contain CRF type-2 receptors. This laboratory previously reported that a CRF receptor antagonist (Rivier et al., 1984), α-helical CRF-(9–41), blocks the CRF-induced increases in $[Ca^{2+}]_i$, with an IC_{50} of 33 nM (Kiang, 1994 and Fig. 3). If sauvagine or urotensin I causes an increase in $[Ca^{2+}]_i$ by interaction with the CRF receptor, then the CRF receptor antagonist should inhibit that increase. To test this possibility, cells were treated with α-helical CRF-(9–41) at different concentrations for 1 min before challenge with either sauvagine or urotensin I. The CRF receptor antagonist blocked the sauvagine- or urotensin I-induced increases in $[Ca^{2+}]_i$ in a concentration-dependent manner, with IC_{50} values of 1.99 ± 0.04 nM and 0.85 ± 0.04 nM, respectively (Fig. 3), suggesting that CRF receptor-operated Ca^{2+} channels are involved.

Previously this laboratory reported that pertussis toxin-sensitive G proteins are involved with the CRF-induced increases in $[Ca^{2+}]_i$ (Kiang, 1994 and Fig. 4). We sought to determine whether similar G proteins are involved in the peptide-induced increase in $[Ca^{2+}]_i$. Cells were treated with 30 ng/ml pertussis toxin for 24 h prior to the challenge with either peptide. This treatment reduced the increases in $[Ca^{2+}]_i$ normally stimulated by sauvagine and urotensin I by 52% and 65%, respectively (Fig. 4). The

Table 1
Inhibition of sauvagine- or urotensin I-induced increases in $[Ca^{2+}]_i$

Treatment	Concentration	Increase in $[Ca^{2+}]_i$ (nM)	
		Sauvagine	Urotensin I
[a] Ca^{2+}	1.6 mM	119 ± 32	143 ± 23
[b] EGTA	10 mM	-45 ± 9 [c]	-14 ± 6 [c]
[a] La^{3+}	10 μM	-48 ± 19 [c]	-29 ± 12 [c]
[a] Verapamil	10 μM	143 ± 34	175 ± 25

Cells were incubated in the various treatment buffers for 1 min prior to the addition of either sauvagine (1 pM) or urotensin I (10 pM).
[a] Buffer contained 1.6 mM Ca^{2+}.
[b] Buffer did not contain Ca^{2+}.
[c] $P < 0.05$ vs. cells treated with the agonist alone in the buffer containing 1.6 mM Ca^{2+}, Student's t-test.

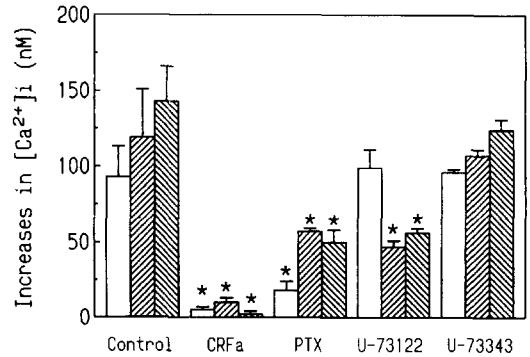

Fig. 4. CRF antagonist, pertussis toxin and U-73122 reduce increases in $[Ca^{2+}]_i$ induced by CRF, sauvagine and urotensin I. Cells were treated with CRF receptor antagonist (CRFa, 50 nM, 1 min), pertussis toxin (PTX, 30 ng/ml, 24 h), U-73122 (1 μM, 1 min), or U-73343 (5 μM, 1 min) before challenge with 42 pM CRF, 1 pM sauvagine, or 10 pM urotensin I ($n = 3$–6). * $P < 0.05$ vs. the respective control, determined by Student's t-test. Open columns: CRF; left-slanted hatched columns: sauvagine; right-slanted hatched columns: urotensin I.

increases in $[Ca^{2+}]_i$ stimulated by sauvagine or urotensin I were also attenuated by treatment with U-73122 (1 μM), an inhibitor of $InsP_3$ production (Bleasdale et al., 1990; Kiang et al., 1994; Kiang and McClain, 1993; Smallridge et al., 1992; Thompson et al., 1991) in the presence of external Ca^{2+} (Fig. 4). U-73343, an inactive analogue of U-73122, was tested to confirm the specificity of the inhibition produced by U-73122. U-73343 up to a concentration of 5 μM increased the resting $[Ca^{2+}]_i$ by 26% in untreated cells but had no effect on the increases in $[Ca^{2+}]_i$ induced by CRF, sauvagine, or urotensin I (Fig. 4), indicating that the inhibitory activity of U-73122 is specific.

These results indicate that the increases in $[Ca^{2+}]_i$ stimulated by the two peptides are derived from two sources, an influx of external Ca^{2+} and a mobilization of Ca^{2+} from an $InsP_3$-sensitive Ca^{2+} pool. The possible involvement of this kind of intracellular Ca^{2+} pool led us to determine whether levels of $InsP_3$ were changed in cells treated with sauvagine or urotensin I.

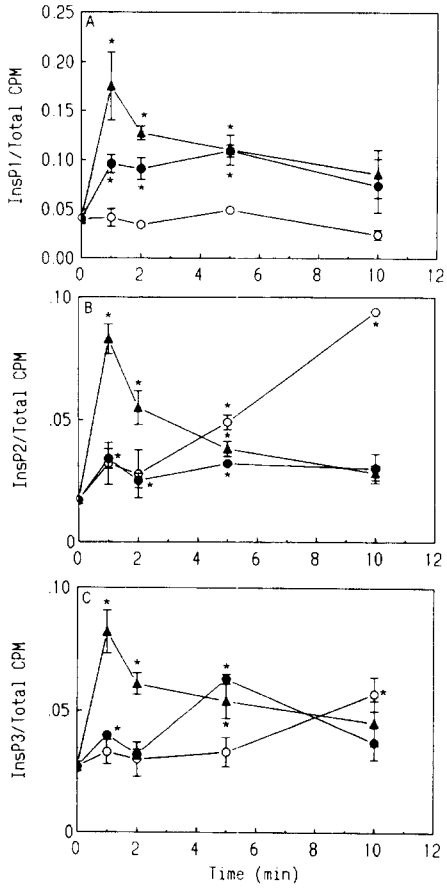

Fig. 5. Time-course of CRF-, sauvagine- and urotensin I-induced increases in $InsP_1$, $InsP_2$ and $InsP_3$. Cells were treated with 42 pM CRF, 1 pM sauvagine, or 10 pM urotensin I for 0, 1, 2, 5 and 10 min. Maximal production occurred after treatment with CRF for 10 min, sauvagine for 1 min and urotensin I for 5 min ($n = 3$). * $P < 0.05$ vs. the 0 time point, determined by one-way ANOVA and studentized-range test. (○) CRF; (▲) sauvagine; (●) urotensin I.

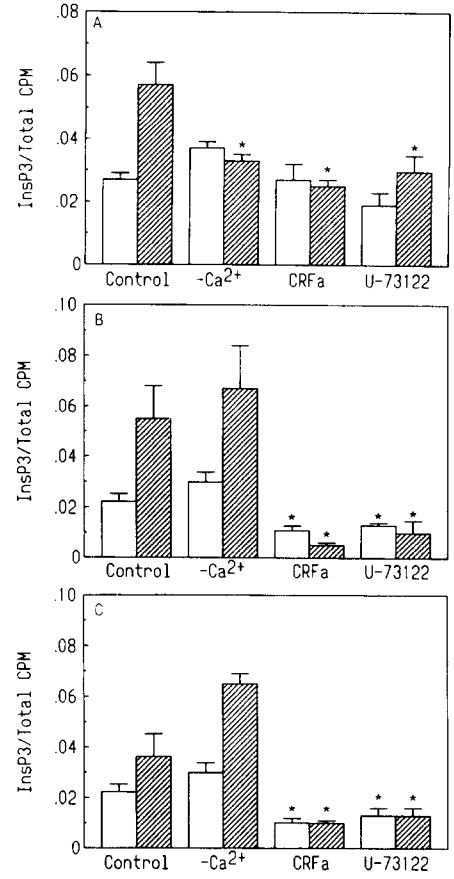

Fig. 6. Increase in $InsP_3$ production induced by CRF, sauvagine and urotensin I is inhibited by CRF receptor antagonist and U-73122 but not by removal of external Ca^{2+}. Cells were treated with 50 nM CRF antagonist (CRFa) or 1 μM U-73122 for 1 min prior to 42 pM CRF for 10 min (A), 1 pM sauvagine for 2 min (B), or 10 pM urotensin I for 5 min (C) in the presence of 1.6 mM Ca^{2+}. The other group of cells were incubated in Ca^{2+}-free buffer containing 10 mM EGTA ($-Ca^{2+}$) for 10 min before challenge with either peptide ($n = 3$). * $P < 0.05$ vs. the respective control, determined by Student's t-test. Open columns: no peptide treatment; hatched columns: peptide challenged.

3.4. Effect of sauvagine and urotensin on $InsP_3$ levels

Previously this laboratory reported that CRF increased $InsP_3$ to maximal levels after a 10 min incubation (Kiang, 1994; Fig. 5). To determine the effect of sauvagine and urotensin I on $InsP_3$ levels, cells were treated with either peptide for 30 s, 1, 2, 5, or 10 min in the presence of external Ca^{2+}. Fig. 5 shows that $InsP_1$, $InsP_2$ and $InsP_3$ increased to maximal levels after a 1 min incubation of sauvagine. Urotensin I also increased levels of inositol phosphates, but the maximal response occurred after 5 min of incubation (Fig. 5). Unlike those observed with CRF, these increases occurred even in the absence of external Ca^{2+}, and they were inhibited by both α-helical CRF-(9–41) and U-73122 (Fig. 6). These observations support the findings from other experiments from this laboratory that indicate the involvement of a CRF receptor and the phospholipase C-mediated process.

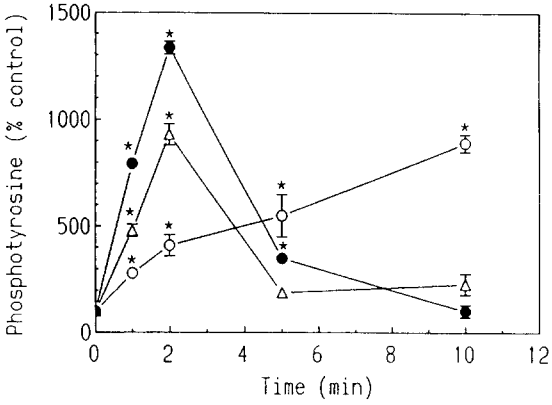

Fig. 7. Increase in protein tyrosine phosphorylation by CRF, sauvagine, and urotensin I. Cells were treated with 42 pM CRF, 1 pM sauvagine, or 10 pM urotensin I for 1, 2, 5 and 10 min in the presence of 1.6 mM Ca^{2+}. Immunoblotting was then performed using an antibody directed to phosphotyrosine. Three independent experiments were conducted. * $P < 0.05$ vs. control, determined by Student's t-test. (○) CRF; (▲) sauvagine; (●) urotensin I.

3.5. Effect of CRF, sauvagine and urotensin I on protein tyrosine phosphorylation

It is known that activation of protein tyrosine kinases (PTKs) increases the phospholipase C activity that leads to increases in $InsP_3$ levels and Ca^{2+} mobilization (Wheeler et al., 1990; Weiss and Littman, 1994). In order to determine whether CRF, sauvagine and urotensin I activate PTKs, A-431 cells were stimulated with the peptides for 1, 2, 5 and 10 min, and levels of protein tyrosine phosphorylation were measured by immunoblotting with an antibody directed against phosphotyrosine. CRF, sauvagine and urotensin I increased the phosphorylation of protein tyrosine. The maximal phosphorylation occurred at 2 min for sauvagine and urotensin I and returned to the basal level at 10 min (Fig. 7). Unlike the more rapid, transient increase induced by these two peptides, CRF increased the phosphotyrosine level gradually for 10 min without a return to baseline values (though longer incubations with CRF were not performed). These results suggest that sauvagine and urotensin I activate PTKs rapidly and CRF activates them slowly.

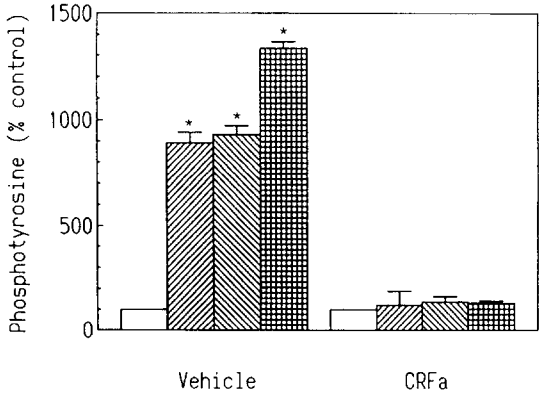

Fig. 8. Inhibition of CRF receptor antagonist on protein tyrosine phosphorylation by CRF, sauvagine, and urotensin I. Cells were treated with 50 nM CRF receptor antagonist (CRFa) prior to CRF (42 pM, 10 min), sauvagine (1 pM, 2 min), or urotensin I (10 pM, 2 min) in the presence of 1.6 mM Ca^{2+}. Immunoblotting was then performed using an antibody directed to phosphotyrosine. Three independent experiments were conducted. * $P < 0.05$ vs. control, determined by Student's t-test. Open columns: control; left-slanted hatched columns: CRF; right-slanted hatched columns: sauvagine; cross-hatched columns: urotensin I.

To determine whether elevated tyrosine phosphorylation was a CRF receptor-mediated process, cells were pretreated with 50 nM α-helical CRF-(9–41) 1 min prior to treatment with the peptides. Fig. 8 shows that the elevation of protein phosphorylation was inhibited by treatment with α-helical CRF-(9–41). The result suggests that the peptide-induced protein tyrosine phosphorylation is mediated through CRF receptors.

4. Discussion

Sauvagine and urotensin I, both of which are highly structurally homologous to CRF, have been shown to have activities similar to CRF in vivo (Lederis et al., 1985; MacCannell et al., 1982; Rivier et al., 1983; Wei and Kiang, 1989). In this study, sauvagine and urotensin I, like CRF, increased $[Ca^{2+}]_i$ in a drug concentration-dependent fashion, demonstrating EC_{50} of 1.4 ± 0.2 and 66 ± 7 fM, respectively. The increases were apparently a result of an influx of extracellular Ca^{2+}, because (1) the increase did not occur if Ca^{2+} was removed from the buffer and (2) the Ca^{2+} channel blocker La^{3+} blocked the increase.

CRF receptors appear to mediate the increase in $[Ca^{2+}]_i$ stimulated by sauvagine and urotensin I, since the increase in $[Ca^{2+}]_i$ induced by the peptides was inhibited by α-helical CRF-(9–41), a CRF receptor antagonist, with IC_{50} values of 1.99 ± 0.04 nM for sauvagine (1 pM) and 0.85 ± 0.04 nM for urotensin I (10 pM). Sauvagine and urotensin I apparently interact only with CRF receptors to increase $[Ca^{2+}]_i$, because incubation of cells with agents that block $[Ca^{2+}]_i$ increases stimulated by other classes of agonist failed to block CRF's effects (Kiang, 1994, 1995).

The Ca^{2+} channels associated with the CRF receptors used by these compounds are not voltage-gated, because the L-type Ca^{2+} channel blocker verapamil failed to block the increase in $[Ca^{2+}]_i$ induced in A-431 cells by sauvagine and urotensin I. This response is different from that observed with CRF and certain other CRF-like peptides. For example, in an earlier study (Kiang, 1995) we found that Ca^{2+} uptake stimulated by the short peptides mystixin-7 and -11 is also mediated by CRF receptors, but occurs through Ca^{2+} channels that are sensitive to verapamil. CRF activates receptors that are also associated with L-type Ca^{2+} channels in human ACTH-secreting pituitary adenoma cells and normal rat small ovoid corticotrophs (Guerineau et al., 1991). The fact that there is a variable

sensitivity to verapamil in A-431 cells treated with different CRF homologues suggests there are different subclasses of type 2 CRF receptors in these cells. It would be extremely interesting to determine whether ACTH-secreting pituitary adenoma cells stimulated with sauvagine and urotensin I demonstrate a similar pattern of verapamil sensitivity.

The coupling of pertussis toxin-sensitive G proteins to CRF receptors is involved in the response of A-431 cells to sauvagine and urotensin I, because treatment with pertussis toxin diminished the sauvagine- and urotensin I-induced increases in $[Ca^{2+}]_i$, a finding similar to that obtained with A-431 cells stimulated with CRF (Kiang, 1994), mystixin-7 and -11 (Kiang, 1995), rat C_6 glioma cells (Lin et al., 1992) and ND8-47 neuroblastoma × dorsal root ganglion hybrid cells (Tang et al., 1995). It should be noted that the direct interaction of sauvagine and urotensin I with receptor-operated Ca^{2+} channels (bypassing the G proteins) cannot be ruled out, because treatment with pertussis toxin was only partially effective at reducing the magnitude of the increase in $[Ca^{2+}]_i$ stimulated by the agents.

Like CRF, both sauvagine and urotensin I increased $InsP_3$. The maximal increase in $InsP_3$ stimulated by these peptides occurred sooner than the 10 min of treatment required with CRF (Kiang, 1994). The increase in $InsP_3$ production stimulated by the peptides did not require external Ca^{2+} and was blocked by a CRF receptor antagonist. This implies that the binding of sauvagine and urotensin I to CRF receptors directly activates G proteins that stimulate the phospholipase C-mediated production of $InsP_3$. CRF receptor antagonist also blocks increases in $InsP_3$ stimulated by CRF, but extracellular Ca^{2+} must be present to observe this effect (Kiang, 1994). This suggests that the increase in $InsP_3$ production stimulated by CRF occurs as a result of CRF receptor-mediated opening of Ca^{2+} channels, which leads to an elevation of $[Ca^{2+}]_i$ that activates phospholipase C. These observed differences between sauvagine and urotensin I vs. CRF reflect possibly different mechanisms of action; sauvagine and urotensin I rapidly stimulated increases in protein tyrosine phosphorylation, whereas CRF did not. An increase in phosphotyrosine levels can occur as a result of activation of PTKs, which has been shown in other systems to be responsible for increases in $InsP_3$ (Weiss and Littman, 1994). The possibility that sauvagine and urotensin I activate the cell in ways different from CRF merely as a consequence of their being used at concentrations that were different multiples of their EC_{50} is not likely, because treatment of cells at concentrations beyond those producing the maximal $[Ca^{2+}]_i$ response does not induce further increases in $[Ca^{2+}]_i$. Furthermore, the amount of the CRF receptor antagonist required to inhibit the agonist-induced increase in $[Ca^{2+}]_i$ is consistent with the potency of each agonist.

Intracellular Ca^{2+} mobilization contributes to the sauvagine- and urotensin I-induced increase in $[Ca^{2+}]_i$, because the increase in $InsP_3$ production that occurred after treatment with the G protein inhibitor pertussis toxin also attenuated the increase in $[Ca^{2+}]_i$. A similar mechanism has been suggested in pituitary cells stimulated with thyrotropin-releasing factor (Smallridge et al., 1992). It does not appear that the increases in $[Ca^{2+}]_i$ stimulated by the peptides is related to increases in the sensitivity of intracellular Ca^{2+} pools because of an increase in their $InsP_3$ receptor levels. No changes in $InsP_3$ receptor 3 levels were observed after stimulation with any of the peptides in this study (Kiang and Wojcikiewicz, unpublished data).

The view that the anti-edema properties of sauvagine, urotensin I and CRF are related to their capacity to increase $[Ca^{2+}]_i$ is supported by the following facts: (1) There is a positive correlation between the ED_{50} values of the peptides for anti-edema and their EC_{50} values for $[Ca^{2+}]_i$ changes (correlation coefficient = 0.99). (2) The relative effectiveness of the three agents to inhibit edema is the same as that for stimulation of $[Ca^{2+}]_i$ changes. (3) Their anti-edema properties and their capacities to increase $[Ca^{2+}]_i$ were blocked by a CRF receptor antagonist, indicating they all use the same receptor to exert their actions. (4) Treatment with pertussis toxin reduced their activities to a similar extent. (5) They all increase $InsP_3$ and mobilize Ca^{2+} from the $InsP_3$-sensitive pools, as shown by the fact that U-73122, an inhibitor of phospholipase C-mediated $InsP_3$ production, inhibited both $InsP_3$ production and the $[Ca^{2+}]_i$ increases induced by these peptides.

The physiological significance of changes in $[Ca^{2+}]_i$ has been extensively documented. It is likely that an increase in $[Ca^{2+}]_i$ serves as a common signal to trigger a variety of cellular functions. For example, in mouse pituitary AtT-20 cells, the increased transcription of c-*fos* mRNA induced by CRF depends on Ca^{2+} (Boutillier et al., 1991). ACTH release in dispersed rat anterior pituitary cells is Ca^{2+}-dependent (Won and Orth, 1990), and the heat shock-induced heat shock protein 72 kDa in human epidermoid A-431 cells is induced by Ca^{2+} ionophores (Ding et al., 1996; Kiang, 1994). It would be of interest to test whether the anti-edema properties of sauvagine and urotensin I result from their capacity to increase $[Ca^{2+}]_i$ in vivo. The excellent correlation between the anti-edema properties of these agents in rats and their capacity to increase $[Ca^{2+}]_i$ in A-431 cells suggests the interesting possibility that cultured A-431 cells might be a useful model to test the potential in vivo effectiveness of other CRF-like peptides.

In summary, this paper demonstrates that sauvagine and urotensin I increased $[Ca^{2+}]_i$ in a concentration-dependent manner. The increase was blocked by removal of extracellular Ca^{2+} or treatment with Ca^{2+} channel blockers, α-helical CRF-(9–41), pertussis toxin, or U-73122. Sauvagine and urotensin I also stimulated $InsP_3$ production, a process that still occurred in the absence of extracellular Ca^{2+} and perhaps is mediated by activation of PTKs. The data

suggest that the increase in $[Ca^{2+}]_i$ is due both to a Ca^{2+} influx through Ca^{2+} channels coupled to CRF receptors and pertussis toxin-sensitive G proteins and an extracellular Ca^{2+}-independent increase in $InsP_3$ that stimulates a mobilization of intracellular Ca^{2+}. Differences between the response of A-431 cells to CRF and CRF-like peptides suggests that different classes of type-2 CRF receptors might exist on these and other cells. This possibility warrants further investigation.

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