

Interleukin-1 inhibits voltage-dependent P/Q-type Ca^{2+} channel associated with the inhibition of the rise of intracellular free Ca^{2+} concentration and catecholamine release in adrenal chromaffin cells

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Abbreviations used: ACh, acetylcholine; CA, catecholamine; CICR, Ca^{2+} -induced Ca^{2+} release; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; E, epinephrine; HPA, hypothalamic-pituitary-adrenal gland; IL-1, Interleukin-1; IL-1RA, IL-1 receptor antagonist; MAP kinases, mitogen-activated protein kinases; NE, norepinephrine; PTX, pertussis toxin; SOC, store-operated Ca^{2+} channel; VOCC, voltage-operated Ca^{2+} channel.

Abstract

Effects of interleukin (IL) on intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) rise and catecholamine (CA) release were examined in isolated, cultured bovine adrenal chromaffin cells. IL-1 α and IL-1 β inhibited the rise of $[\text{Ca}^{2+}]_i$ and CA release induced by acetylcholine (ACh) and excess KCl both in normal and in Ca^{2+} -sucrose medium. IL-1 receptor antagonist, IL-1RA by pretreatment blocked the inhibitory actions of IL-1. IL-1 α reduced CA release induced by veratridine in normal medium but not in the presence of diltiazem. Analysis using specific blockers for voltage-operated Ca^{2+} channels revealed that IL-1 α specifically inhibited the P/Q-type Ca^{2+} channel to reduce $[\text{Ca}^{2+}]_i$ rise induced by excess KCl. IL-1 did not affect $[\text{Ca}^{2+}]_i$ rise induced either by bradykinin or caffeine in Ca^{2+} -deprived medium or via activation of store-operated Ca^{2+} channel. The inhibitory effects of IL-1 were blocked by pretreatments of cells with herbimycin A, U0126 and PD 98054, but not with SB202190, SP 600125 or pertussis toxin (PTX), inhibited the induction of the inhibitory action of IL-1.

These results demonstrated that IL-1 inhibits stimulation-evoked $[\text{Ca}^{2+}]_i$ rise and CA release in chromaffin cells by blocking voltage-operated P/O-type Ca^{2+} channels. The inhibitory action of IL-1 may be mediated through tyrosine kinase and MEK/ERK pathways.

Introduction

The most prominent neuroendocrine system for host defense against diseases and stress is activation of the hypothalamic-pituitary-adrenal gland (HPA) axis, resulting in the release of adrenocorticotrophic hormone and glucocorticoids. Cytokines are the most potent regulators of the HPA axis [1, 2]. In addition to this axis, catecholamine (CA) release from adrenal medulla also forms an important mechanism for host defense against diseases and stress. The secretory response of CA in adrenal medullary chromaffin cells comes under central control via the splanchnic nerve. It receives peripheral regulation (such as auto-regulation) by various factors which are co-released with CA from chromaffin cells, and circular regulation by factors in the blood which increase or decrease in response to diseases and stress [3]. Cytokines are induced in circulation under some conditions, including severe trauma and fever [4-9]. Interleukin (IL)-1 α has been localized immunohistochemically to peripheral neurons with a distribution pattern similar to that of noradrenergic fibers [10, 11] and in rat adrenal medulla where its expression is altered by physiological stimulation of adrenomedullary chromaffin cells [12]. Induction of IL-1 like substances in PC 12 cells by NGF [13] and induction of IL-1 α mRNA by various stimulations in bovine adrenal chromaffin cells [14] and in PC 12 cells [15], and the release of IL-1 from bovine adrenal chromaffin cells [16] have been reported. These results suggest that cytokines may play a physiological role in adrenal medullary functions. In support of this idea, cytokines have been shown to differentially regulate neuropeptide biosynthesis in bovine adrenal chromaffin cells [17]. Furthermore, Yanagihara et al. [18] reported that

long-time exposure of bovine adrenal chromaffin cells with IL-1 β increased CA release from the cells. Therefore, it is assumed that cytokines directly regulate the process leading to the exocytotic release of CA from adrenomedullary chromaffin cells. To address this issue, we examined the effect of cytokines on intracellular Ca²⁺ dynamics and CA release in adrenal medullary chromaffin cells. It was found that IL-1 produced a significant inhibitory effect on stimulation-induced increase in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) and release of CA by selectively inhibiting voltage-dependent P/Q-type Ca²⁺ channels.

1. Materials and methods

2.1. Materials

The drugs used were recombinant human IL-1 α , human IL-1 β , human IL-1RA, (R&D systems Inc., Minneapolis, MN, USA); bradykinin, ω -agatoxin IVA, ω -conotoxin GVIA and ω -conotoxin MVIIC (Peptide Institute Inc., Osaka, Japan); caffeine, digitonin, herbimycin A and pertussis toxin (Wako Pure Chemicals Industries, Osaka, Japan); diltiazem hydrochloride, nicardipine hydrochloride, nifedipine, thapsigargin and veratridine (Sigma, St. Louis, MO, USA); PD 98059 [2'-amino-3'-methoxyflavone], SB 202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole], SP600125 [Anthra[1,9-*cd*]pyrazol-6(2*H*)-one; JNK inhibitor II] and U0126 [1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene] (Calbiochem, LaJolla, CA, USA) and fura-2 acetoxymethyl

ester (fura-2/AM) (Dojindo Chemicals, Kumamoto, Japan). All other chemicals were reagent grade and purchased from commercial sources specified elsewhere. Nicardipine and veratridine were dissolved in ethanol. Diltiazem, fura-2/AM, herbimycin A, nifedipine, PD 98059, SB 202190, SP600125 and U0126 were dissolved in dimethyl sulfoxide and diluted appropriately (final concentration of ethanol and dimethyl sulfoxide was 0.1%).

2.2. Cell preparation and culture

Chromaffin cells of bovine adrenal glands were isolated enzymatically according to the procedure described by [19], with some modifications [20, 21]. Cells were maintained in Dulbecco's modified Eagle's medium containing 10 % fetal calf serum, penicillin G 100 units/ml, streptomycin 100 µg/ml, ascorbate 0.1 mM, and HEPES 5 mM for 24 - 72 h at 37 °C under 5 % CO₂/95 % air as suspension culture for measurements of [Ca²⁺]_i, or as monolayer culture on 35 mm tissue culture Petri dish (5x10⁵ cells/ml) for 2 - 5 days for CA release assay. Cells were washed and suspended before use in a medium containing NaCl 150; KCl 5; MgSO₄ 1; CaCl₂ 1.3; glucose 5; HEPES-Tris buffer 10 (in mM) and bovine serum albumin (BSA) 0.5 %, pH 7.4 or Ca²⁺-sucrose medium containing CaCl₂ 1.3 mM, sucrose 340 mM HEPES-Tris 10 mM and BSA 0.1 %, pH 7.35.

2.3. Measurements of [Ca²⁺]_i

For the measurement of $[Ca^{2+}]_i$, cells were incubated at 32 °C with 1 μ M fura-2 AM for 30 min in order to load the dye. Cells were then centrifuged at 15 g for 10 min and resuspended to yield 3×10^6 cells/ml. Fluorescence was measured using a dual wavelength, fluorescence spectrophotometric mode, with an excitation of 340 and 380 nm, and an emission of 510 nm as described previously [22]. $[Ca^{2+}]_i$ was calculated from the fluorescent ratio at 340 and 380 nm, using the equation of Grynkiewicz et al. [23] and a value of 224 nM for the K_d of fura-2.

2.4. Measurement of catecholamine (CA) release

To measure CA release from permeabilized chromaffin cells, cells in monolayer culture were permeabilized with 10 μ M of digitonin in a Ca^{2+} -free KGP-solution (150 mM glutamate potassium salt, 10 mM PIPES, 5 mM nitrilotriacetic acid, 4.5 mM magnesium acetate, pH 7.0) containing 5 mM Mg-ATP, 0.5 mM EGTA and 0.1 % BSA as described previously [22]. Cells were then incubated for 20 min with KGP-solution containing various amounts of calcium chloride to yield the indicated free Ca^{2+} concentrations. After the incubation, the medium was immediately separated from the cells, then perchloric acid was added (5 % of final concentration) and the mixture was centrifuged at 4,500 g for 15 min. IL-1s were added 40 min before the permeabilization with digitonin.

CA release from intact cultured bovine chromaffin cells was performed as previously described [20, 21]. Cultured bovine adrenal chromaffin cells were incubated for 30 min with or without 100 pg/ml IL-1 α . The medium was replaced with fresh medium containing

IL-1 α and ACh, excess KCl at the concentrations indicated, then further incubated for 9 min at 37 °C. The total CA content of the clear supernatant was measured fluorometrically by the method of von Euler and Lishajko [24] with adrenaline as a standard. Values were represented as net increase in CA release (stimulated release – corresponding basal release).

The determination of the ratio of NE/(NE + E) in the medium was performed using HPLC-ED as previously described Sakuma et al. [25].

2.5. Statistics

All experiments were carried out in triplicate, and each experiment was repeated at least 3 times. Data obtained are expressed as mean \pm SEM. Statistical significance between means for comparison of two variables was estimated using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Inhibition by IL-1 of stimulation-evoked CA release

Pretreatment of cultured bovine adrenal chromaffin cells with 100 pg of IL-1 α had little effect on basal CA release (Basal CA release during 30 min incubations were 0.15 ± 0.01 [control] and 0.16 ± 0.01 $\mu\text{g}/10^6$ cells [100 pg IL-1 α], respectively, n=25) and significantly inhibited stimulation-induced CA release (Table 1). IL-1 α reduced even the maximal release induced by 30 μM ACh and 75 mM KCl. In Ca $^{2+}$ sucrose medium, 25 mM KCl induced larger CA release than in normal medium. IL-1 α reduced CA release in this condition to an extent similar to the normal medium. Veratridine, a Na $^{+}$ -ionophore, caused CA release by activating Na $^{+}$ -Ca $^{2+}$ exchange through the plasma membrane and voltage-operated Ca $^{2+}$ channel (VOCC), a result of the cytosolic accumulation of Na $^{+}$ in chromaffin cells [26, 27]. IL-1 α reduced veratridine-induced CA release but this effect was lost in the presence of diltiazem, an inhibitor of VOCC. IL-1 α had no effect on Ca $^{2+}$ -induced CA release from digitonin-permeabilized cells. IL-1 receptor antagonist, IL-1RA blocked the inhibitory effect of IL-1 α by pretreatment of cells with IL-1RA. However, when cells were treated with IL-1RA at 30 min after the treatment of IL-1 α then incubated for 90 min, the inhibitory effect of IL-1 α on ACh-induced CA release was not recovered. Basal release in intact cells with the normal and Ca $^{2+}$ -Sucrose solutions and permeabilized cells (at 10 nM free Ca $^{2+}$ concentration) were 0.08 ± 0.01 , 0.55 ± 0.02 and 0.17 ± 0.02 $\mu\text{g}/10^6$ cells, respectively. Basal CA release was not altered by the presence of the drugs tested and/or IL-1 α . The effects of IL-1 α on CA content and the ratio of released norepinephrine (NE)

and epinephrine (E) were examined. Total CA in cells treated with or without IL-1 α , 100 pg/ml for 0, 30, 60 and 120 min was 59.5 ± 4.3 , 60.8 ± 3.3 , 60.0 ± 4.9 , 63.4 ± 5.8 and 58.3 ± 1.2 , 59.7 ± 1.3 , 56.1 ± 1.4 , 56.2 ± 1.0 $\mu\text{g}/10^6$ cells, respectively. The ratios of NE/(NE + E) release for 30 min in the resting state were 0.30 ± 0.03 (control) and 0.33 ± 0.04 (100 pg/ml IL-1 α), and 0.21 ± 0.02 (5 μM ACh) and 0.21 ± 0.03 (100 pg/ml IL-1 α + 5 μM ACh), respectively. The ratios in response to 30 μM ACh and 25 mM KCl were also not altered by IL-1 α . Thus, the content of CA and the ratio of NE and E release were not altered by IL-1 α , suggesting that IL-1 α inhibit the synthesis of neither NE nor E. IL-1 β had similar effects on CA release (data not shown).

Inhibition of $[\text{Ca}^{2+}]_i$ rise by IL-1

Stimulated CA release from bovine adrenal chromaffin cells is entirely dependent on the presence of extracellular Ca^{2+} . To evaluate the mechanism of IL-1 α -induced inhibition of CA release, the effect of IL-1 α on Ca^{2+} dynamics was examined. Typical patterns of stimulation-induced $[\text{Ca}^{2+}]_i$ rise and the effects of IL-1 α are shown in Fig.1. IL-1 α alone had little effect on basal $[\text{Ca}^{2+}]_i$ during the 10 min assay (Fig. 1A-a). IL-1 α reduced the peak and the following sustained $[\text{Ca}^{2+}]_i$ rise induced by excess KCl (Fig. 1A-b).

To examine the effect of IL-1 α on the mobilization of Ca^{2+} from intracellular Ca^{2+} stores induced by such mechanisms as inositol trisphosphate (IP_3)-mediated release and Ca^{2+} -induced Ca^{2+} release (CICR), We analyzed the effects of IL-1 α on bradykinin- and caffeine-induced $[\text{Ca}^{2+}]_i$ rise in the absence of Ca^{2+} in the medium. Bradykinin has been

shown to increase the production of IP₃ and increase Ca²⁺ mobilization from IP₃-sensitive stores in chromaffin cells [29]. Caffeine also mobilizes Ca²⁺ by sensitizing CICR mechanisms [22, 30-32]. IL-1 α had no effect on either bradykinin- or caffeine-induced [Ca²⁺]_i rise in the absence of Ca²⁺ in the medium (Fig. 1B-a,b). Effect of IL-1 on Ca²⁺ influx through store-operated Ca²⁺ channel (SOC) [28] was also examined (Fig. 1B-c). Thapsigargin, which is an inhibitor of Ca²⁺-ATPase, depletes stored Ca²⁺ without involving plasma membrane receptor activation. Thus it is widely used to activate SOC. In chromaffin cells in this model, IL-1 α had no effect either on thapsigargin-induced [Ca²⁺]_i rise in Ca²⁺ free condition or on the large increase in [Ca²⁺]_i by the addition of Ca²⁺ into the medium after thapsigargin treatment (Fig.1B-c).

Fig. 2 shows the concentration-dependent effects of IL-1 α and IL-1 β on stimulation-evoked [Ca²⁺]_i rise. Both cytokines significantly inhibited [Ca²⁺]_i rise induced by excess KCl or ACh at 1 pg/ml with maximal reduction by about 40 % at 10 pg/ml. Increasing concentration of IL-1 α up to 10 ng/ml induced no further increase in the inhibitory effects (Fig. 2). The inhibitory effects of IL-1 α , IL-1 β on [Ca²⁺]_i rise and on CA release appeared after a latent period of several minutes and intensified as a function of incubation time (data not shown).

Inhibition of VOCC by IL-1

In agreement with the restoration of the inhibitory effect of IL-1 α on CA release in Ca²⁺-sucrose medium (Table 1), IL-1 α reduced 17 mM KCl-induced [Ca²⁺]_i rise in Ca²⁺-sucrose

medium where the VOCC is the main route for the increase in $[Ca^{2+}]_i$ as shown by the blockade of 17 mM KCl-induced $[Ca^{2+}]_i$ rise by diltazem, an inhibitor of VOCC (Fig. 3). Taken together with the disappearance of the inhibitory effect of IL-1 α on veratridine-induced CA release in the presence of diltiazem, these findings suggest that VOCC is involved at the site of IL-1 α action.

Adrenal chromaffin cells develop several types of VOCC; P/Q type, N-type and L-type. As VOCC was the suggested site of inhibition for IL-1 α by the results presented in Table 1 and Fig. 1, it is important to determine the type of VOCC that the target for IL-1s. This issue was addressed using specific VOCC inhibitors. ω -Conotoxin GVIA (N-type channel blocker) had little effect on excess KCl-induced $[Ca^{2+}]_i$ rise, suggesting that the contribution of the N-type channel is small. ω -Conotoxin GVIA had little effect on the inhibitory effect of IL-1 α . ω -Agatoxin IVA (P/Q-type channel blocker) reduced both the peak rise and sustained $[Ca^{2+}]_i$ rise, suggesting that the P/Q-type channel significantly contributes to the rise of $[Ca^{2+}]_i$. In the presence of ω -agatoxin IVA, the inhibitory effect of IL-1 α was abolished. ω -Conotoxin MVIIC (N/P/Q-type blocker) also reduced the excess KCl-induced $[Ca^{2+}]_i$ rise and abolished the inhibitory effect of IL-1 α . Nicardipine (L-type channel blocker) significantly reduced the excess KCl-induced peak $[Ca^{2+}]_i$ rise and attenuated the sustained rise close to the basal level, suggesting that the L-type channel plays a major role in the excess KCl-induced $[Ca^{2+}]_i$ rise. In the presence of nicardipine with or without of ω -conotoxin GVIA, the $[Ca^{2+}]_i$ rise induced by excess KCl was further reduced by IL-1 α . In the presence of nicardipine and ω -agatoxin IVA, $[Ca^{2+}]_i$ rise was minimal and was not affected by IL-1 α (Fig. 3). Therefore, the remaining $[Ca^{2+}]_i$ rise in the

presence of nicardipine and ω -conotoxin GVIA could be due to the P/Q-type channel and IL-1 α inhibits this channel. Thus, the blockers of the P/Q type channel, ω -agatoxin IVA or ω -conotoxin MVIIC, presented alone or in any combination with other blockers prevented the inhibitory effect of IL-1 α on excess KCl-induced $[Ca^{2+}]_i$ rise. Similar results were obtained using IL-1 β (Fig. 3), and ACh as a stimulant (data not shown). These results clearly demonstrated that IL-1 inhibited specifically the P/Q type Ca^{2+} channel.

In agreement with the effects of these VOCC inhibitors on $[Ca^{2+}]_i$ rise, CA release evoked by excess KCl was significantly reduced by ω -agatoxin IVA and ω -conotoxin MVIIC, and more profoundly by nicardipine and nifedipine, but not by ω -conotoxin GVIA (Table 2). The basal values of CA release in the presence of drugs tested were 0.08 ± 0.01 ; ω -agatoxin IVA, 0.09 ± 0.01 ; ω -conotoxin GVIA, 0.09 ± 0.01 ; ω -conotoxin MVIIC, 0.10 ± 0.01 ; ω -agatoxin IVA + ω -conotoxin GVIA, 0.09 ± 0.01 ; nicardipine, 0.09 ± 0.01 ; nifedipine, 0.1 ± 0.01 $\mu\text{g}/10^6$ cells, respectively. The inhibitory effect of IL-1 α on CA release was also abolished in the presence of the blockers of the P/Q type channel (ω -agatoxin IVA and ω -conotoxin MVIIC) (Table 2). IL-1 α still produced the inhibitory effect on excess KCl-evoked CA release even in the presence of nicardipine or nifedipine until CA release was profoundly blocked by these inhibitors. Basal CA release in the presence of the drugs tested was not altered by IL-1 α .

3.3. Effects of inhibitors of signaling on IL-1-induced inhibition of $[Ca^{2+}]_i$ rise

Although the intracellular signal transduction systems coupled to IL-1 receptors are still uncertain, Plata-Salamán and French-Mullen [33] reported that the depression of Ca^{2+}

channel current in hippocampal neurons by IL-1 β was prevented by inhibitors of protein kinase C and suggested the involvement of protein kinase C. In the present study, the inhibitors for protein kinase C, staurosporine and A kinase, H-89 had no influence on IL-1 α -induced inhibition of secretory response (data not shown). In cells pretreated with an inhibitor of tyrosine kinase, herbimycin A, IL-1 α lost its inhibitory effect on excess KCl-evoked [Ca²⁺]_i rise. Involvement of mitogen-activated protein kinases (MAP kinases) in the production of the inhibitory effect of IL-1 on excess KCl-evoked [Ca²⁺]_i rise were examined using the inhibitors. MEK/ERK inhibitors, U0126 and PD 98059 significantly attenuated the effect of IL-1. U0126 and PD 98059 increased basal [Ca²⁺]_i. p38 inhibitor, SB 202190 and JNK inhibitor, SP600125 did not affect on the effect of IL-1. An inhibitor of Gi/Go protein coupled pathway, pertussis toxin (PTX) had no effect on the inhibitory effect of IL-1 (Table 3).

Discussion

Intraventricular injections of cytokines transiently increase blood CA [34]. Yanagihara et al. [18] reported that when bovine adrenal chromaffin cells were cultured with IL-1 for 24 hrs, a small amount of CA was released into the medium. Another study reported that IL-1 and IL-2 did not stimulate epinephrine release from porcine chromaffin cells [35]. The present study demonstrated noteworthy inhibitory effects of IL-1 α and IL-1 β on [Ca²⁺]_i rise and CA release in cultured bovine chromaffin cells. The effects were blocked by the

endogenous IL-1 receptor inhibitory protein, IL-1RA. Thus, IL-1s may interact with the type 1 IL-1 receptor expressed in the chromaffin cells to produce the inhibitory effects.

The mechanism of IL-1-induced inhibition of CA release does not seem related to inhibition of CA biosynthesis because the total CA content and the ratio of NE/(NE + E) were not altered by IL-1. $[Ca^{2+}]_i$ rise is essential for stimulation-secretion coupling in adrenal chromaffin cells. The IL-1-induced inhibition of $[Ca^{2+}]_i$ rise evoked by excess KCl and ACh could be a reliable cause of the IL-1 inhibition of the stimulation-evoked secretion. The sources of Ca^{2+} for the $[Ca^{2+}]_i$ rise in response to physiological stimulation in adrenal chromaffin cells are the influx of Ca^{2+} through plasma membrane via VOCC, nicotinic ACh receptor and SOC, and the mobilization of Ca^{2+} from intracellular Ca^{2+} stores induced by such mechanisms as IP_3 -mediated release and CICR. Since IL-1 decreased the $[Ca^{2+}]_i$ rise evoked not only by ACh but also by excess KCl depolarization, it is unlikely that the inhibitory effects of IL-1 are due to modification of nicotinic ACh receptor functions. The evidence that the inhibitory effect of IL-1 α on veratridine-induced CA release was lost in the presence of the VOCC blocker, diltiazem, suggests that IL-1 blocks VOCC. This idea is reinforced by the observation that IL-1 α decreased the KCl-induced $[Ca^{2+}]_i$ rise even in a Ca^{2+} -sucrose medium, which rules out any involvement of an effect on the Na^+ channel. Moreover, there is some evidence that IL-1 suppresses ion channel activities. For examples, IL-1 β depresses the voltage-activated inward current of the identified central neurons of *Helix pomatia* [36], the voltage-gated Ca^{2+} channel currents in guinea-pig hippocampal CA1 neurons [33], L- and N-type Ca^{2+} channel activity in rat cortical neurons [37] and L-type Ca^{2+} current activated by adrenergic receptor

stimulation in rat ventricular myocytes [38]. Based on the effect of IL-1 on bradykinin- and caffeine-induced $[Ca^{2+}]_i$ rise and on thapsigargin-induced Ca^{2+} entry, it does not seem that $[Ca^{2+}]_i$ rise mediated by IP_3 , CICR and SOC is involved in the IL-1-induced inhibition of $[Ca^{2+}]_i$ rise. In addition, IL-1 had no effect on exocytosis triggered by Ca^{2+} , evaluated by CA release induced by the direct increase in Ca^{2+} concentration in medium in digitonin-permeabilized cells. Therefore, we conclude that VOCC is a critical site for IL-1 action in decreasing $[Ca^{2+}]_i$ rise, resulting in the inhibition of CA release.

To identify which type of Ca^{2+} channels are involved in the inhibitory effect of IL-1 on $[Ca^{2+}]_i$, the effects of specific blockers for L-, N- and P/Q-type channels on the effect of IL-1 α were examined. The inhibitory effect of IL-1 α was abolished in the presence of only the blockers of the P/Q-type channel (ω -agatoxin IVA or ω -conotoxin MVIIC) suggesting that IL-1 α specifically inhibits the P/Q-type Ca^{2+} channel. It has been demonstrated that Q-type Ca^{2+} channels are coupled more tightly to active exocytotic sites than L-type channels [39]. Actually, inhibitors for P/Q-type, but not N-type VOCC, reduced CA release induced by excess KCl, although the secretory response in adrenal chromaffin cells is predominantly dependent on L-type VOCC. Therefore, the inhibition of P/Q-type VOCC by IL-1s can be reflected in the reduction of CA release. It is interesting to consider the physiological regulation by IL-1 on CA release in human adrenal chromaffin cells where P/Q-type Ca^{2+} channels are primarily expressed [40]. Also, P/Q-type channels are developed in brain and motor nerve endings. Several diseases, such as episodic ataxia type 2; EA2, familial hemiplegic migraine [41] and spinocerebellar ataxia type 6; SCA6 [42] and Lambert-Eaton Myasthenia syndrome (LEMS) [43], are due to dysfunction of P/Q-type channels in the

brain. Whether or not IL-1 modifies P/Q-type Ca^{2+} channels in nervous systems as a physiological and/or pathological state remains to be elucidated but is a matter of keen interest.

One persistent aspect of IL-1 signal transduction is the activation of MAP-kinases, which are very rapid cellular responses, and it represents a point of convergence in intracellular signaling activated by tyrosine kinases, by protein kinase C and A kinase [44]. Although the importance of MAP kinase pathway in the regulation of gene expression is well appreciated, it contributes to other forms of short-term, post-translational modulation. A recent study showed that IL-1 β enhanced NMDA receptor function through activation of Src tyrosine kinases and subsequent phosphorylation of NMDA receptor subunit NR2A/B [45]. The present observations that a tyrosine kinase inhibitor, herbimycin, MEK/ERK inhibitors, U0126 and PD 98059, but not p38 inhibitor, SB 202190, JNK inhibitor, SP600125 and Gi/Go protein inhibitor, PTX, inhibited the induction of the inhibitory action of IL-1 suggest that IL-1 may regulate P/Q type VOCC through a tyrosine kinase-, MEK/ERK-mediated pathway. MEK/ERK inhibitors by themselves increased basal $[\text{Ca}^{2+}]_i$, suggesting that this signaling pathway negatively regulate P/Q type VOCC. Taken together, IL-1 may activate MEK/ERK pathway to facilitate negative regulation of P/Q type channels. Actually we observed previously that IL-1 α increases p42/44 MAP kinase in chromaffin cells (unpublished observation) and thus a possibility may arise that P/Q channel activity is regulated by phosphorylation of the channel by MAP kinases. Whether the inhibitory effect of IL-1 is mediated through the direct phosphorylation of P/Q channel

and/or associated proteins or the synthesis of proteins that interact with the channel remains for further study.

The presence and release of IL-1 in adrenal chromaffin cells have been demonstrated [12-16]. Thus, it is possible that IL-1 released from chromaffin cells produces a feedback inhibition of CA release from the cells. Currie et al. [46] reported that resident macrophages in the adrenal gland release unidentified messengers that inhibit *ICa* in the chromaffin cell, suggesting paracrine signaling between macrophage and chromaffin cell Ca^{2+} channels. The blood level of IL-1 increases under such circumstances as infection or severe trauma to something on the order of pg/ml, though circulating IL-1 is normally very low [4-9]. In acute toxicity caused by clinically administered cytokines for the treatment of cancer or chronic infections, IL-1 causes significant, long lasting hypotension [47-49]. The evidence demonstrated from the present study may provide the possibility that IL-1 plays a signaling role in these physiological and pathological conditions.

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Figure Legends

Fig.1. Effects of IL-1 α on stimulation-induced rise of [Ca²⁺]_i in cultured bovine adrenal chromaffin cells. Following 30 min pretreatment with or without 100 pg/ml IL-1 α in the dark at 32 °C, cells were washed rapidly with fresh medium to remove extracellular (leaked) dye before the fluorescence measurement. They were then incubated for 3 min followed by the indicated stimulants (A, B). The cells were exposed to Ca²⁺-deficient medium (containing 0.1 mM EGTA), B-a,b,c for 3 min before challenge with the indicated stimulants. CaCl₂ (1.3 mM) were added 9 min after extracellular Ca²⁺ deprivation (B-c). The results of a typical experiment are shown (gray traces are in the absence of IL-1 α , (A-a, B)). The arrows on the traces indicate the addition of appropriate stimulants. Experiments were repeated with at least three different batches of cells with similar results.

Fig. 2. Inhibitory effects of IL-1 α and IL-1 β on [Ca²⁺]_i rise induced by excess KCl or ACh in bovine adrenal chromaffin cells. Concentration-response curve for the effects of IL-1 α and IL-1 β on stimulation-induced rise of [Ca²⁺]_i. Experimental design was similar to those described in Fig. 1. Values are the mean \pm SEM of the peak rise of [Ca²⁺]_i (n= 4 to 20). Significantly different from the corresponding control at * p<0.05, ** p<0.01.

Fig. 3. Effects of various voltage sensitive Ca²⁺ channel inhibitors on IL-1 inhibition of stimulation-evoked [Ca²⁺]_i rise in cultured bovine adrenal chromaffin cells. Following 30 min pretreatment with or without 100 pg/ml IL-1 α and IL-1 β , cells were exposed to Ca²⁺-sucrose medium or normal medium with or without various voltage sensitive Ca²⁺ channel

inhibitors for 3 min before challenge with 17 mM KCl. The results of a typical experiment are shown (gray traces are in the absence of IL-1). The arrows on the traces indicate the addition of 17 mM KCl. Experiments were repeated with at least three different batches of cells with similar results. ω -AgTx IVA, ω -agatoxin IVA; ω -CgTx GVIA, ω -conotoxin GVIA; ω -CgTx MVIIC, ω -conotoxin MVIIC

Table 1. Inhibition of stimulation-evoked CA release induced by IL-1 α from cultured bovine adrenal chromaffin cells

Stimulants	Preincubation	Increase in CA release ($\mu\text{g}/10^6$ cells)			
		- IL1 α	+ IL-1 α (100 pg/ml)		
Exp.1 Intact cells					
ACh	3 μM	0.36 \pm 0.09	0.22 \pm 0.08*		
	5 μM	1.16 \pm 0.13	0.68 \pm 0.09*		
	10 μM	1.98 \pm 0.15	1.32 \pm 0.10*		
	30 μM	2.80 \pm 0.14	1.74 \pm 0.10*		
	100 μM	2.64 \pm 0.17	1.79 \pm 0.12*		
KCl	20 mM	0.42 \pm 0.02	0.22 \pm 0.04*		
	25 mM	0.90 \pm 0.08	0.56 \pm 0.07*		
	50 mM	1.59 \pm 0.07	1.15 \pm 0.05*		
	75 mM	1.61 \pm 0.10	1.16 \pm 0.08*		
KCl	25 mM	Vehicle	0.99 \pm 0.08		
		Ca ²⁺ -Sucrose solution	1.23 \pm 0.12		
Veratridine	25 μM	Vehicle	0.74 \pm 0.08		
		Diltiazem 30 μM	0.45 \pm 0.06		
Exp.2 Digitonin-permeabilized cells					
Free Ca ²⁺ concentration					
100 nM		0.73 \pm 0.08	0.77 \pm 0.08		
Exp.3 Intact cells					
Stimulant	Preincubation 1		Preincubation 2		
ACh	5 μM	Vehicle	Vehicle ^a	1.30 \pm 0.04	0.84 \pm 0.05*
	5 μM	Vehicle	IL-1RA 100 ng/ml ^b	1.28 \pm 0.06	1.26 \pm 0.07
	5 μM	IL-1 α 100 pg/ml	Vehicle ^c	0.83 \pm 0.05	0.85 \pm 0.04
	5 μM	IL-1 α 100 pg/ml	IL-1RA 100 ng/ml ^d	0.84 \pm 0.05	0.84 \pm 0.02

Exp. 1. Cultured bovine adrenal chromaffin cells were incubated for 30 min with or without 100 pg/ml IL-1 α . The medium was replaced with fresh medium containing corresponding IL-1 α and ACh, excess KCl at the concentrations indicated, then further incubated for 9 min at 37 °C. Diltiazem was added 3 min before challenge with veratridine.

Exp. 2. Following 30 min pretreatment with or without 100 pg/ml IL-1 α , cells were permeabilized for 5 min with 10 μM digitonin in Ca²⁺-free KGP medium. Extracellular fluids were replaced with fresh medium containing the indicated concentration of free Ca²⁺.

Exp. 3. Cells were preincubated, (a) without IL-1 α (preinc. 1) and without IL-1RA (preinc. 2), (b) without IL-1 α (preinc. 1) and with IL-1RA (preinc. 2), (c) with IL-1 α (preinc. 1) and without IL-1RA (preinc. 2), (d) with IL-1 α (preinc. 1) and with IL-1RA (preinc. 2). Preincubation 1 and 2 were performed for 30 and 60 min, respectively. After the preincubation 2, all cells were challenged with ACh as in Exp. 1. Basal release in intact cells with normal solution or Ca²⁺-Sucrose solution and permeabilized cells (at 10 nM free Ca²⁺ concentration) were 0.08 \pm 0.01, 0.55 \pm 0.02 and 0.17 \pm 0.02 $\mu\text{g}/10^6$ cells, respectively. The basal CA release were not altered by the presence of drugs tested and/or IL-1 α .

Values are the mean \pm SEM of the net increase in CA release attained with four samples in a batch. Significantly different from the corresponding control at * p<0.01.

Table 2. Effects of various types of voltage-operated Ca²⁺ channel antagonists on IL-1 α induced inhibition of excess KCl-evoked CA release from cultured bovine adrenal chromaffin cells.

Addition	KCl (25 mM)-induced CA release ($\mu\text{g}/10^6$ cells)	
	- IL1 α (100 pg/ml)	+ IL1 α (100 pg/ml)
None	0.74 \pm 0.08	0.49 \pm 0.04*
ω -AgTx IVA 0.3 μM	0.44 \pm 0.06	0.40 \pm 0.05
ω -CgTx GVIA 1 μM	0.68 \pm 0.07	0.45 \pm 0.04*
ω -CgTx MVIIC 1 μM	0.42 \pm 0.06	0.39 \pm 0.03
ω -AgTx IVA + ω -CgTx GVIA	0.38 \pm 0.05	0.37 \pm 0.05
None	0.94 \pm 0.04	0.70 \pm 0.03*
Nicardipine 3 nM	0.75 \pm 0.01	0.63 \pm 0.02*
30 nM	0.44 \pm 0.01	0.39 \pm 0.01*
300 nM	0.18 \pm 0.02	0.15 \pm 0.01
None	0.93 \pm 0.02	0.75 \pm 0.01*
Nifedipine 10 nM	0.70 \pm 0.03	0.56 \pm 0.02*
100 nM	0.35 \pm 0.01	0.27 \pm 0.01*
1 μM	0.18 \pm 0.01	0.12 \pm 0.01
3 μM	0.12 \pm 0.01	0.09 \pm 0.01

Experimental design was similar to those described in table 1. Ca²⁺ channel antagonists were pretreated for 3 min before challenge with KCl. Values are the mean \pm SEM of the net increase in CA release attained with four samples in a batch. The basal CA release in the presence of drugs tested (basal values were 0.08 \pm 0.01; ω -AgTx IVA, 0.09 \pm 0.01; ω -CgTx GVIA, 0.09 \pm 0.01; ω -CgTx MVIIC, 0.10 \pm 0.01; ω -AgTx IVA + ω -CgTx GVIA, 0.09 \pm 0.01; nicardipine, 0.09 \pm 0.01; nifedipine, 0.1 \pm 0.01 $\mu\text{g}/10^6$ cells, respectively). The basal CA release in the presence of drugs tested were not altered by IL-1 α Significantly different from the corresponding control at * p<0.01. ω -AgTx IVA, ω -agatoxin IVA; ω -CgTx GVIA, ω -conotoxin GVIA; ω -CgTx MVIIC, ω -conotoxin MVIIC

Table 3. Effects of tyrosine kinase inhibitor, specific inhibitors of MEK/ERK, p38 or Jun kinase pathway and pertussis toxin (PTX) on IL-1 α induced inhibition of excess KCl-evoked [Ca²⁺]_i rise in cultured bovine adrenal chromaffin cells

Stimulants	Preincubation	[Ca ²⁺] _i rise (nM)		
		- IL-1 α	+ IL-1 α (100 pg/ml)	Ratio (+IL-1 α / -IL-1 α)
KCl 17 mM	Vehicle	212.3 \pm 7.9	135.2 \pm 6.1**	0.636 \pm 0.014
	Herbimycin A 1 μ M	237.7 \pm 1.0	220.0 \pm 3.5	0.925 \pm 0.017 ^{##}
KCl 17 mM	Vehicle	231.2 \pm 6.9	157.2 \pm 5.8**	0.678 \pm 0.012
	PD 98059 30 μ M	347.8 \pm 33.1	308.9 \pm 12.7	0.898 \pm 0.035 ^{##}
		60 μ M	464.9 \pm 38.3	401.1 \pm 31.4*
	U0126 10 μ M	303.2 \pm 11.2	277.8 \pm 17.5*	0.898 \pm 0.020 ^{##}
		25 μ M	419.9 \pm 16.2	398.1 \pm 16.7
	SB202190 1 μ M	258.3 \pm 2.7	169.5 \pm 3.3**	0.658 \pm 0.011
		10 μ M	202.1 \pm 13.7	154.9 \pm 7.6**
	SP 600125 1 μ M	217.9 \pm 13.3	162.9 \pm 9.4**	0.710 \pm 0.025
10 μ M		206.9 \pm 14.9	163.4 \pm 9.6**	0.755 \pm 0.029
KCl 17 mM	Vehicle	220.9 \pm 6.3	150.3 \pm 5.8**	0.686 \pm 0.026
	PTX 20 ng/ml	207.6 \pm 3.9	142.5 \pm 2.6**	0.690 \pm 0.017

Experimental design was similar to those described in table 1 and Fig. 1. Herbimycin A, was added 90 min before the addition of IL-1 α . PD 98059, U0126, SB 202190, and SP 600125 were added 30 min before the addition of IL-1 α . Pertussis toxin (PTX) was added 12 hr before the addition of IL-1 α . Values are the mean \pm SEM of the peak rise of [Ca²⁺]_i (n=5 to 9). The basal [Ca²⁺]_i in the presence of drugs tested (basal values were 87.6 \pm 1.5; herbimycin A, 89.9 \pm 6.1; PD 98059, 109.9 \pm 9.4; U0126, 86.4 \pm 2.7; SB202190, 82.5 \pm 4.6; SP 600125, 108.5 \pm 2.8; PTX, 109.5 \pm 2.3 nM, respectively). The basal [Ca²⁺]_i in the presence of drugs tested were not altered by IL-1 α . Significantly different from the corresponding control (-IL-1 α) at * p<0.05, ** p<0.01; Significantly different from the corresponding control ratio (+IL-1 α / -IL-1 α) at [#]p<0.05, ^{##}p<0.001.

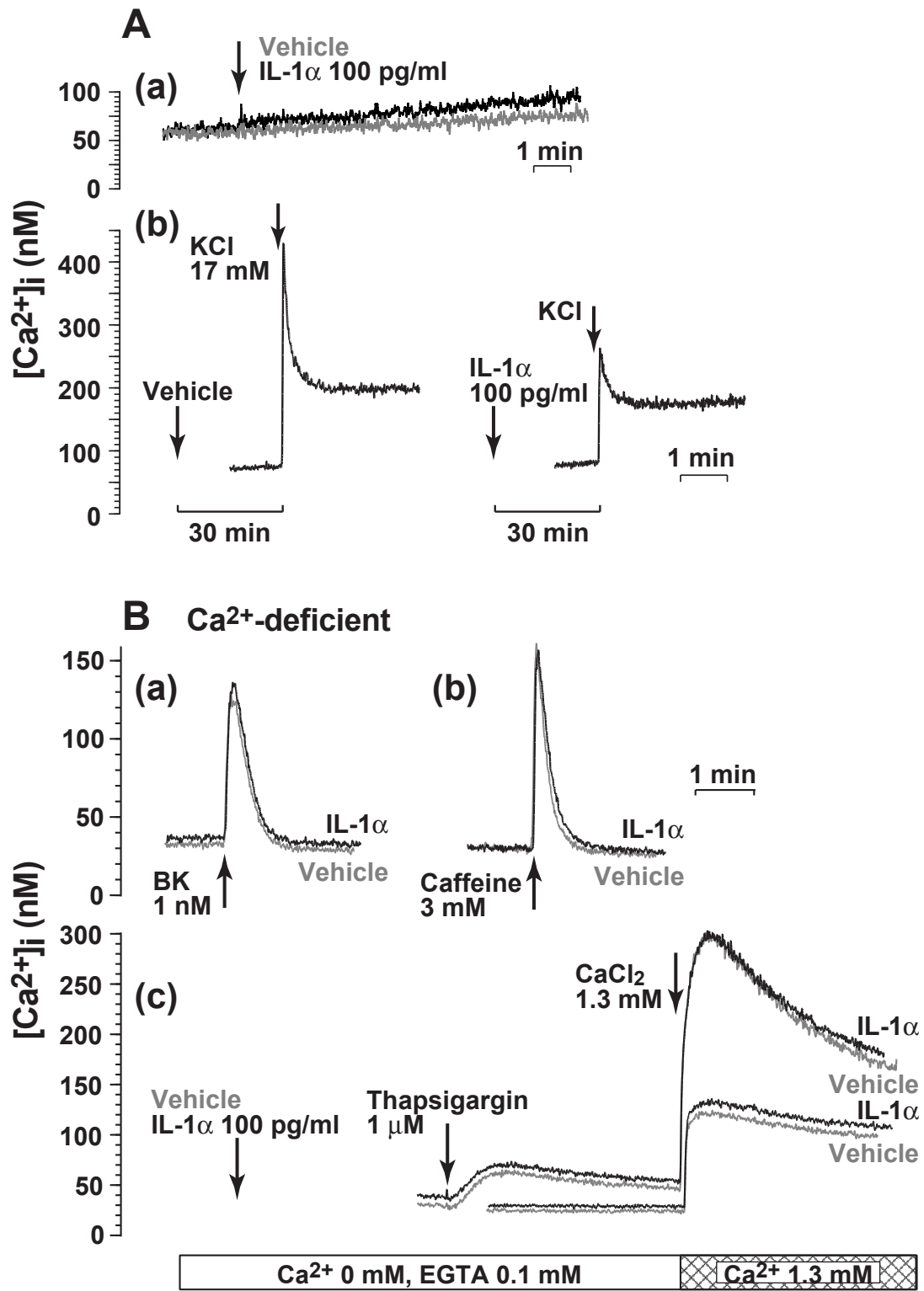


Fig. 1.

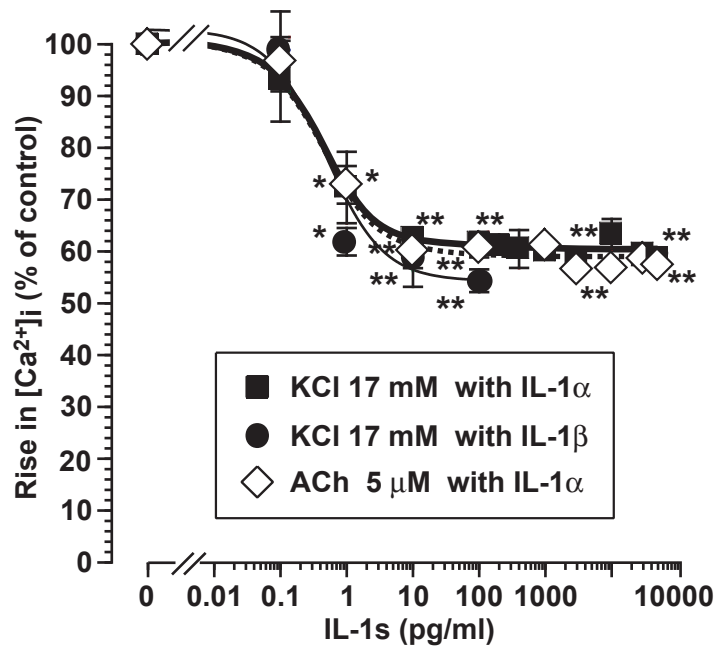


Fig. 2.

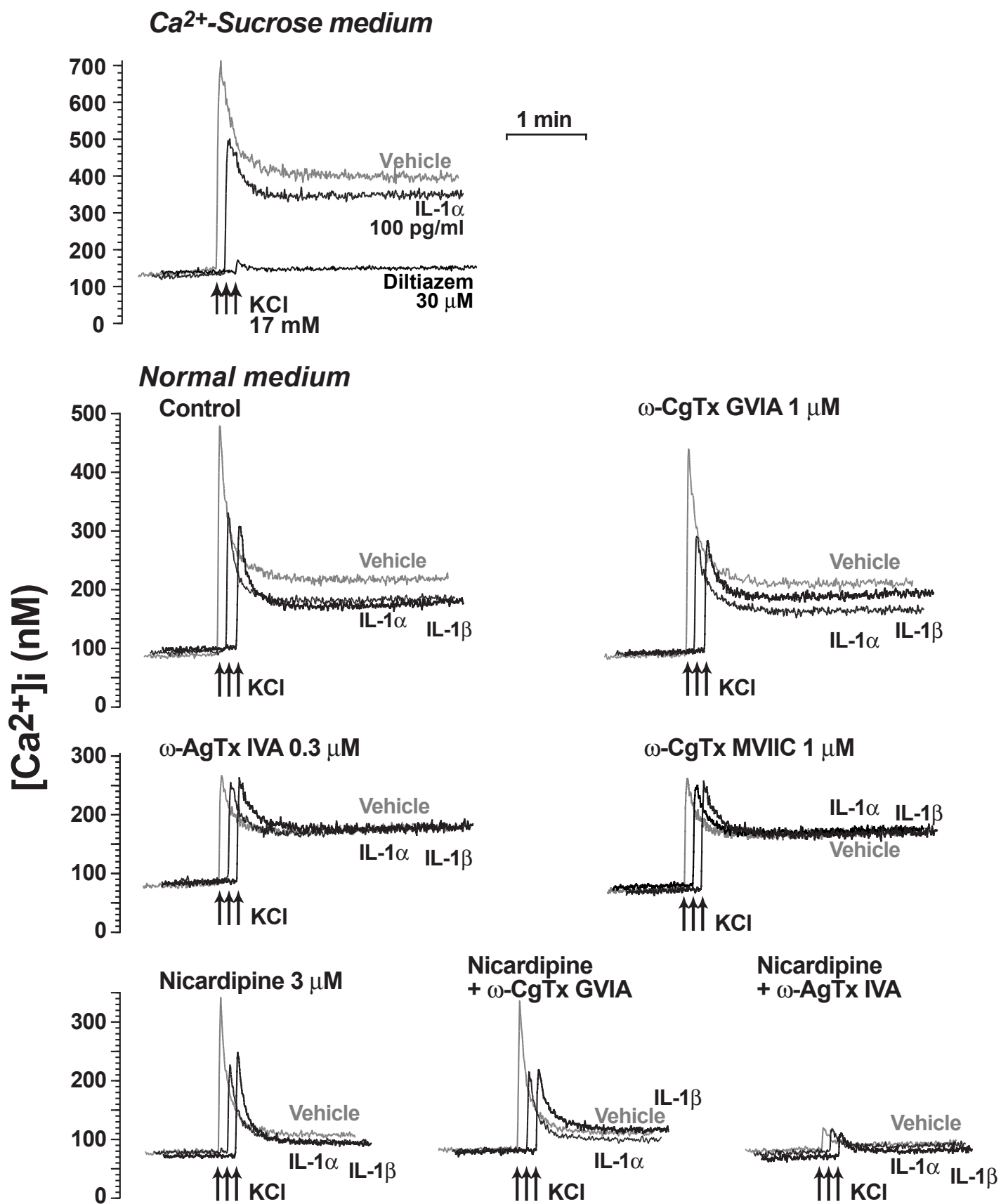


Fig. 3.