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SHORT COMMUNICATION

Ca^{2+} Entry Induced by Calcium Influx Factor and Its Regulation by Protein Kinase C in Rabbit Neutrophils

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ABSTRACT. Extracellular application of acid extract from platelet-activating factor- or thapsigargin-treated rabbit neutrophils induced a rise of cytosolic free calcium concentration ($[Ca^{2+}]_i$) in neutrophils and adrenal chromaffin cells suspended in Ca^{2+}-containing, but not in Ca^{2+}-deficient, medium. The ability of the extract to selectively induce Ca^{2+} entry was also confirmed by the increase in $^{45}Ca^{2+}$ uptake and failure to stimulate Ca^{2+} release in digitonin-permeabilized neutrophils. 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibited the extract-induced $[Ca^{2+}]_i$ rise in a staurosporine (ST)-sensitive fashion, neither of which had any effect on its production. SK&F 96365 and econazole also reduced extract-induced Ca^{2+} entry. These results suggest that a Ca^{2+} entry-inducible substrate (calcium influx factor) is extracted from Ca^{2+} store-depleted neutrophils, and that its action may be regulated by protein kinase C and certain pharmacological agents. BIOCHEM PHARMACOL 52;1:167–171, 1996.

KEY WORDS. calcium influx factor; cytosolic free calcium; protein kinase C; neutrophils; calcium entry; platelet-activating factor

It is well recognized that an increase in $[Ca^{2+}]_i$§ is one of the key steps for intracellular cell signalling in a variety of cells. Release of Ca^{2+} from intracellular Ca^{2+} stores and the influx of Ca^{2+} across the plasma membrane are the necessary events for a rise in $[Ca^{2+}]_i$. Activation of receptors coupled with phosphatidylinositide hydrolysis frequently exhibits a biphasic increase in $[Ca^{2+}]_i$ (i.e. a transient initial peak rise followed by a sustained rise). Such biphasic responses are initially sustained by inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release from intracellular Ca^{2+} stores and, thereafter, by Ca^{2+} influx across the plasma membrane. The latter is thought to be an important process in cell activation, especially in nonexcitable cells such as neutrophils. However, neither the structural molecules nor the mechanism for activating and regulating Ca^{2+} influx are certain, and specific blockers of Ca^{2+} entry that would elucidate its function and clinical importance have not yet been obtained, in contrast to various antagonists of voltage-operated Ca^{2+} channels (VOC).

It has been proposed that the depletion of Ca^{2+} stores by physiological stimulation or pharmacological manipulation induces Ca^{2+} entry: this is called SDCE [1]. In fact, it is reported that the filling state of Ca^{2+} stores regulates Ca^{2+} entry in neutrophils [2], leukemia cells [3], and many other cell types. Although the mechanisms underlying SDCE have been the subject of intensive investigation, very little is known about the signalling that occurs between the empty/full state of Ca^{2+} stores and the activity of cell surface ion channels.

Recently Parekh *et al.* [4] suggested that Ca^{2+} entry activated by IP_3 pool depletion in Xenopus oocytes is related to phosphatases and an unidentified diffusible messenger(s). Randriamampita and Tsien [5] showed the presence of a diffusible messenger produced and released by the depletion of Ca^{2+} stores of Jurkat T-lymphocytes to activate Ca^{2+} entry. The messenger is a novel, small, negatively-charged molecule they called CIF and suggested as a probable messenger of SDCE. Thomas and Hanley also reported that CIF-like substances were extracted from thapsigargin-treated Jurkat cells that induce Ca^{2+} entry when applied internally in Xenopus oocytes, and that the activity is potentiated by a protein phosphatase inhibitor, okadaic acid [6]. Although this evidence suggests that CIF may be an elusive cellular messenger for Ca^{2+} entry, questions concerning CIF as a physiological messenger of SDCE have also been raised [7]. The present study was undertaken to elucidate whether or not CIF is produced in neutrophils and whether or not Ca^{2+} entry induced by CIF is regulatable by PKC, given that SDCE in neutrophils is negatively controlled by PKC [8, 9]. The effects of a putative Ca^{2+} entry blocker on CIF-induced Ca^{2+} entry were also examined.

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§*Abbreviations:* $[Ca^{2+}]_i$, cytosolic free calcium concentration; PAF, platelet-activating factor; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; Tgg, thapsigargin; SDCE, Ca^{2+} store depletion-induced Ca^{2+} entry; CIF, calcium influx factor.

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MATERIALS AND METHODS

Neutrophils and adrenal chromaffin cells were prepared as described previously [9, 10]. Methods for measurement of $[Ca^{2+}]_i$ in intact cells or the release of Ca^{2+} in digitonin-permeabilized cell were conducted as described previously [9]. Statistical analyses were performed by calculating the integration of the area over basal level in the patterns of $[Ca^{2+}]_i$ rise by a computer [9] with the students *t*-test.

Crude extracts were prepared from neutrophils stimulated in a Ca^{2+}-free medium by the procedure reported by Randriamampita and Tsien [5]. Briefly, isolated neutrophils suspended in Ca^{2+}-free Hank's balanced salt solution (HBSS) (2–5 × 10^7 cells/mL) were incubated with 1 μM Tgg or 10 nM PAF for 2 min and centrifuged (180 *g*, 10 min). The pellet was resuspended in 700 μL of Ca^{2+}-free HBSS plus 150 μL 1 M HCl. After 20 min, the suspension was centrifuged and the supernatant brought back to pH 7.3 by adding 1 M NaOH (final volume 1 mL). The supernatant was kept with 2 units/mL hexokinase for 20 min and frozen at −20°C until use. For partial purification of CIF, aliquots of the extract were spotted on silica gel plates (Silica Gel 60; Merk, Darmstatdt, Germany). TLC was performed using a solvent system of chloroform/methanol/water (65:35:6, by vol.). Silica gel areas were scraped off in a 1 cm area of the spot and each fraction reextracted in acid solvent; lipid-soluble materials were extracted using the procedure of Bligh and Dyer [11]. The extracts were evaporated under an N_2 gas stream, dissolved in 1 mL HBSS, and submitted to $[Ca^{2+}]_i$ measurement.

A $^{45}Ca^{2+}$ uptake assay was conducted as follows. Isolated neutrophils at 10^7 cells/tube were preincubated at 37°C for 5 min, and uptake was then initiated by the addition of $^{45}CaCl_2$ (2 mCi/mL) with or without 10 μL extract or 10 nM PAF. After incubation, the uptake was terminated by adding 3 mL of ice-cold Ca^{2+}-free HBSS, followed by rapid filtration and washing with 3 mL ice-cold Ca^{2+}-free HBSS under vacuum on Whatman glassfiber filters GF/C. The radioactivity in the filters was determined by liquid scintillation counting.

The reagents used were PAF and econazole (SIGMA, St. Louis, MO, U.S.A.), hexokinase and staurosporine (ST) (Boehringer, Mannheim, Germany), Tgg and TPA (Funakoshi, Tokyo, Japan), fura-2/AM (Dojindo Chemicals, Kumamoto, Japan), $^{45}CaCl_2$ (0.85–1.35 GBq/mg Ca, Amersham, Bucks, U.K.), polymorphprep (Nycomed Pharma AS, Oslo, Norway), BN50739 (Institut Henri Beaufour, Le Plessis Robinson, France) and SK&F 96365 (SmithKline Beecham, Herts, U.K.).

RESULTS AND DISCUSSION

Acid extract was prepared from rabbit neutrophils incubated with PAF or Tgg in Ca^{2+}-deficient medium according to the procedure described by Randriamampita and Tsien [5]; its effects on $[Ca^{2+}]_i$ in rabbit neutrophils are shown in Fig. 1. The extract from PAF-treated cells induced a slow rise in $[Ca^{2+}]_i$ after a 4–6-sec time lag, an increase not inhibited by the PAF receptor antagonist BN50739 (Fig. 1A-2). Conversely, the PAF-induced rapid rise in $[Ca^{2+}]_i$ was blocked by BN50739 (Fig. 1A-1). The extract from Tgg-treated neutrophils also induced $[Ca^{2+}]_i$ elevation in neutrophils (Fig. 1A-2), but the extract from nonstimulated cells had no effect on $[Ca^{2+}]_i$ (Fig. 1A-3). During $[Ca^{2+}]_i$ elevation following addition of Tgg, additional Tgg caused no further rise in $[Ca^{2+}]_i$ but the extract increased $[Ca^{2+}]_i$

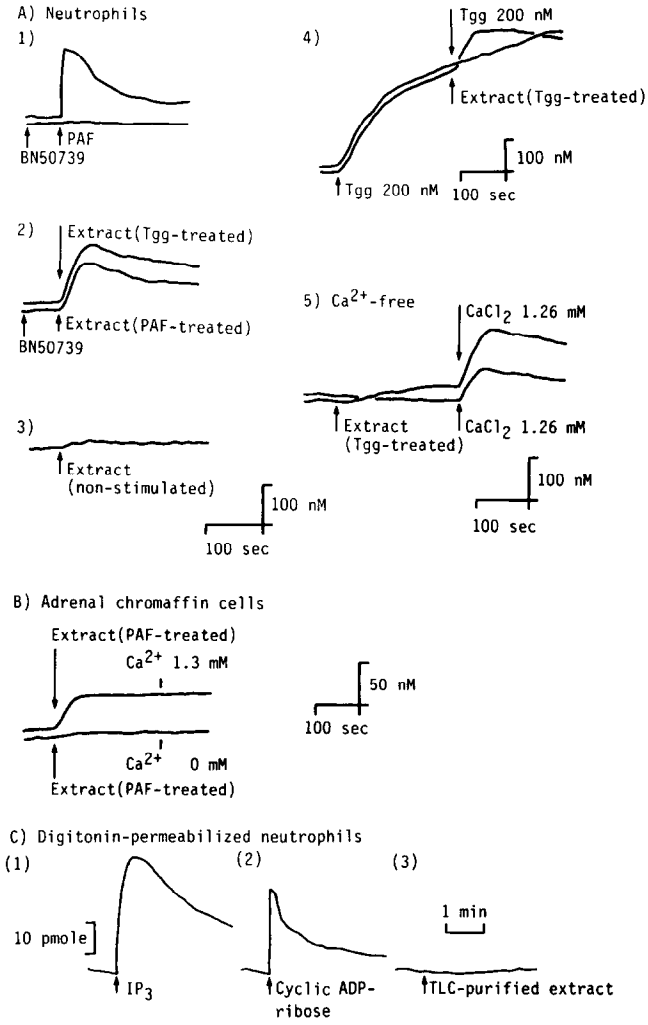

FIG. 1. Typical patterns of $[Ca^{2+}]_i$ rise induced by PAF and the extract from PAF- and thapsigargin (Tgg)-treated cells with or without extracellular calcium in rabbit neutrophils (A) and in bovine adrenal chromaffin cells (B). Cells were suspended in normal HBSS or Ca^{2+}-free medium (Ca^{2+} was replaced with an equivalent amount of sucrose). BN50739 (1 μM) was added 1 min before stimulation with PAF (0.1 pM) or 10 μL of 1 mL extract prepared from 3 × 10^7 cells. (C) Release of Ca^{2+} from permeabilized neutrophils by digitonin. IP_3 (75 nM), cyclic ADP-ribose (500 nM), and extract (20 μL) were added in digitonin-permeabilized cells.

(Fig. 1A-4), suggesting that it is unlikely that the rise in $[Ca^{2+}]_i$ induced by the extract is due to the contamination with PAF or Tgg in the extract. It has been reported that several types of Ca^{2+} stores are present in numerous types of cells, such as IP_3-sensitive stores and IP_3-insensitive and ryanodine-sensitive stores; the former is sensitive to Tgg, but the latter is resistant to Tgg [12]. It has also been reported that CIF may be composed of not a single, but several components [6]. Thus, the extract may contain several factors that activate Ca^{2+} influx in Tgg-treated cells. TLC analysis also demonstrated increased $[Ca^{2+}]_i$ activity of the extract, which was recovered from the original spotting area in the solvent system and was separated from authentic PAF (Rf = 0.21) and Tgg (Rf = 0.58), where the activity for the $[Ca^{2+}]_i$ rise was not detected (data not shown). The treatment of cells with the TLC-purified extract from Tgg-treated cells in the Ca^{2+}-free medium induced only a slight rise in $[Ca^{2+}]_i$, but produced a most significant rise in $[Ca^{2+}]_i$ when Ca^{2+} was added to the medium (Fig. 1A-5) (the integrated area of $[Ca^{2+}]_i$ increase over the basal level for 180 sec from the addition of Ca^{2+} to Tgg-treated cells calculated by a computer from the figure was 175.7 ± 4.9% of that of untreated cells, $P < 0.05$, $n = 3$). All these results suggest a Ca^{2+} entry-inducible substrate(s) extracted from the Ca^{2+} store-depleted neutrophils that we termed the CIF, as Randriamampita and Tsien [5] did previously.

In bovine adrenal chromaffin cells, the $[Ca^{2+}]_i$ rise due to acetylcholine (ACh) is thought to be mainly due to the influx of Ca^{2+} through nicotinic ACh receptors and VOC. A SDCE-dependent pathway is also suspected of participating in the $[Ca^{2+}]_i$ rise. In addition, PAF by itself induces no elevation of $[Ca^{2+}]_i$ in chromaffin cells [10]. Thus the effect of the extract from PAF-treated neutrophils on chromaffin cells was examined. The extract from neutrophils induced a $[Ca^{2+}]_i$ rise in a Ca^{2+}-containing medium, but not in a Ca^{2+}-free medium (Fig. 1B). Therefore, the extract is active in different cell types where the rise of $[Ca^{2+}]_i$ induced by the extract is dependent on extracellular Ca^{2+}. It seems unlikely that the extract mobilizes Ca^{2+} from the intracellular Ca^{2+} stores: the application of the extract in digitonin-permeabilized neutrophils did not cause Ca^{2+} release, and IP_3, a well-known agonist for Ca^{2+} release, and cyclic ADP-ribose, a novel candidate for endogenous ryanodine receptor agonist, both stimulated Ca^{2+} release (Fig. 1C). In addition, the extract increased $^{45}Ca^{2+}$ uptake into neutrophils time-dependently (Fig. 2). The stimulation of $^{45}Ca^{2+}$ uptake by the extract at 30 sec corresponded to PAF (10 nM)-induced $^{45}Ca^{2+}$ uptake. These results further support the notion that the extract contains a Ca^{2+} entry-inducible substrate.

The novel diffusible messenger called CIF has been reported to have hydroxyls on adjacent carbons, a phosphate, and a Mr under 500 by chemical characterization [5]. We reproduced a similar chemical characterization of the extract from Tgg-treated neutrophils (data not shown). In-

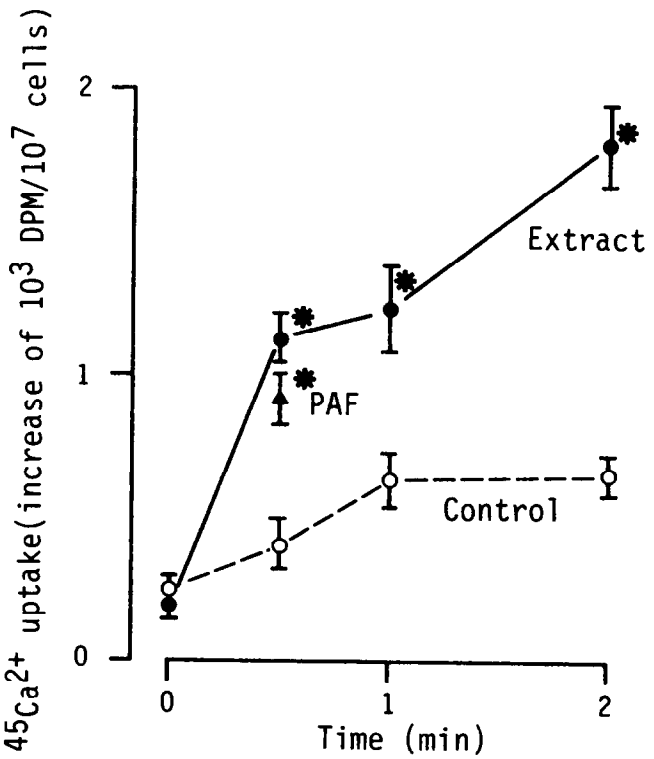

FIG. 2. $^{45}Ca^{2+}$ uptake in neutrophils stimulated with the extract from thapsigargin-treated cells and PAF. Cells were incubated with the extract (●), PAF (▲) and vehicle (○) for 30 sec, 1 min, and 2 min as described in Methods. *Significant increase over control, $P < 0.05$, n = 5.

hibitors of protein phosphatases, such as okadaic acid or cyclosporin A, potentiate SDCE or the $[Ca^{2+}]_i$ rise induced by CIF in several types of cells [4, 6, 13], and CIF degradation in cell-free homogenates of lymphocytes is prevented by these inhibitors [6, 13]. However, the evidence could not explain how extracellularly applied extract or CIF exerts its activity. One may assume that such substances go through the plasma membrane by any mechanism and are released into the extracellular space at the time of their production [5]. The time lag required for the extract to induce a $[Ca^{2+}]_i$ rise in the present study might be due to its passing through the plasma membrane. The molecular species of CIF, its pathways of synthesis and degradation, as well as its mechanism of action remain to be defined after it is accepted as an intracellular, physiological messenger.

In contrast to the considerable progress made in elucidating the mechanism of entry of Ca^{2+} *via* VOC with the help of available high-affinity ligands, including organic "calcium antagonists" and toxins, specific inhibitors of SDCE are not available. Recent reports have shown several compounds that may have an inhibitory effect on SDCE. SK&F 96365 has been shown to inhibit receptor-stimulation-mediated Ca^{2+} entry induced in human platelets and neutrophils, although it also inhibited VOC [14]. Cytochrome P-450 inhibitors, such as econozole, have also been

shown to inhibit SDCE in human neutrophils [2] and rat thymocytes [15]. Examining whether or not such compounds affect CIF-induced Ca^{2+} entry may very well help in confirming that CIF is a messenger of SDCE and in developing inhibitors of SDCE. Given concentrations of SK&F 96365 and econazole did reduce extract-induced $[Ca^{2+}]_i$ rise (SK&F 96365: 52.7 ± 1.8% of control, econazole: 64.9 ± 1.3% of control, $P < 0.05$, $n = 3$) (Fig. 3a, b).

Protein kinase C (PKC) is a key enzyme that plays a crucial role in the regulation of numerous cellular activations in neutrophils. It has been shown that TPA inhibits Ca^{2+} entry induced by Tgg in human neutrophils [8], in thyroid FRTL-5 cells [16], and in human leukemia HL-60 cells [17], and that induced by PAF and Tgg in rabbit neutrophils [9]. These results support the hypothesis that PKC negatively regulates SDCE, and suggest that CIF-induced Ca^{2+} entry could also be regulated negatively by PKC. The results of the present study clearly show that TPA inhibited $[Ca^{2+}]_i$ elevation induced by the extract from Tgg-treated cells (the integrated area of the TPA-treated sample was 0%) and that the inhibitory effect of TPA was attenuated by staurosporine (ST) (the integrated area of the ST + TPA-treated sample was 49.7 ± 1.6% of the control, $P < 0.05$, $n = 3$) (Fig. 3c). ST itself had no effect on the extract-induced $[Ca^{2+}]_i$ rise (95.3 ± 4.6% of the control). Both of the extracts from cells treated with Tgg in the presence of TPA or ST induced a $[Ca^{2+}]_i$ elevation similar to that induced by the control extract from cells treated with Tgg alone (TPA: 97.2 ± 2.4% of the control, ST: 83.3 ± 1.5% of the control) (Fig. 3d). These results suggest that Ca^{2+} entry induced by the Ca^{2+} entry-inducible substrate might be regulated negatively by PKC after the substrate is produced.

In summary, the present results demonstrate that the extract from PAF- and Tgg-treated cells acts as a Ca^{2+} entry-inducible substrate, called a CIF, and suggest that CIF-mediated Ca^{2+} entry is regulated negatively by PKC at a step distal to the production of CIF.

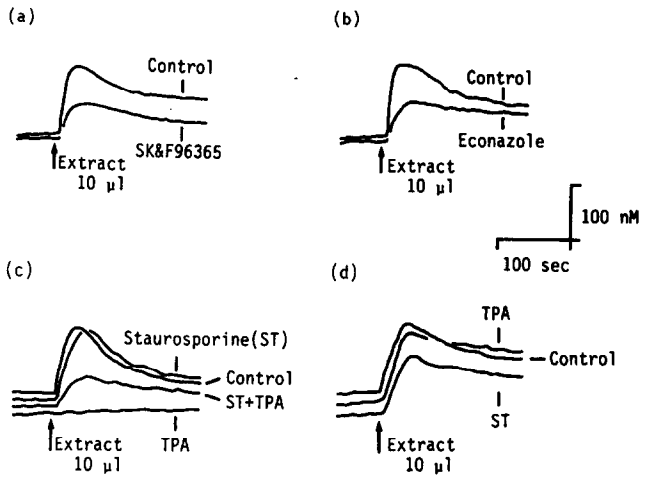

FIG. 3. Effects of SK&F 96365 (a) and econazole (b) on the $[Ca^{2+}]_i$ rise induced by the extract from Tgg-treated cells. Effects of protein kinase C activator and inhibitor on the $[Ca^{2+}]_i$ rise induced by the extract from Tgg-treated cells (c) and on the production of the Ca^{2+} entry-inducible substrate (d). (a and b): Cells were incubated with 50 µM SK&F 96365 or 20 µM econazole for 5 min before extract stimulation. (c): Cells were incubated at 37°C with 10 nM TPA for 1 min and/or 10 nM staurosporine (ST) for 5 min before the addition of extract (10 µL). (d): Cells pretreated with 10 nM TPA for 1 min and/or 10 nM ST for 10 min were stimulated with 200 nM Tgg in Ca^{2+}-free medium and extraction was then conducted. The procedures are described in Methods. The extracts were submitted to $[Ca^{2+}]_i$ measurement.

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