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Short communication

Spontaneous Mn^{2+} entry is specifically inhibited by calyculin A in human leukemic HL-60 cells

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Abstract

The effects of calyculin A and other agents which enhance protein Ser/Thr phosphorylation, on the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and spontaneous Mn^{2+} entry were investigated in fura-2-loaded human leukemic HL-60 cells. Calyculin A (30 nM), a specific inhibitor of protein Ser/Thr phosphatase (PP) 1 and 2A, significantly decreased $[Ca^{2+}]_i$. By contrast, another structurally unrelated inhibitor of PP1 and 2A, okadaic acid (1 μM), caused a slight elevation in $[Ca^{2+}]_i$. Forskolin (30 μM), which could enhance protein kinase A activity by raising cAMP concentration, also caused a rise in $[Ca^{2+}]_i$. Phorbol myristate acetate (PMA, 300 nM), an activator of protein kinase C, did not have a significant effect on $[Ca^{2+}]_i$. Spontaneous entry of Mn^{2+} (a surrogate ion for Ca^{2+}) was strongly inhibited by calyculin A, but not okadaic acid, forskolin or phorbol myristate acetate. Such inhibition was not significantly affected by staurosporine (300 nM), a non-specific inhibitor of protein Ser/Thr kinases. Our results suggest that calyculin A inhibited a plasmalemmal leak pathway to Mn^{2+} (and Ca^{2+}), probably leading to a decrease in $[Ca^{2+}]_i$. Inhibition of spontaneous Mn^{2+} entry by calyculin A may depend on a specific protein phosphorylation pattern induced by staurosporine-insensitive protein kinase(s). © 1997 Elsevier Science B.V.

Keywords: Calyculin A; Ca^{2+}, cytosolic; Mn^{2+}; Protein phosphatase; Protein phosphorylation; HL-60 cell

1. Introduction

The degree of protein phosphorylation depends on the relative activities of protein kinases and phosphatases. An enhancement of protein phosphorylation can therefore be induced either by activation of protein kinases or inhibition of protein phosphatases. In the latter case, the attenuation of protein phosphatase action indirectly causes the enhancement of protein kinase activities. A number of agents have been known to potently and selectively inhibit protein Ser/Thr phosphatases 1 and 2A (PP1 and 2A). One of these is calyculin A, a metabolite isolated from the marine sponge *Discodermia calyx* (Kato et al., 1986; Ishihara et al., 1989a). This compound causes hyperphosphorylation of proteins in various cell types (Murphy and Westwick, 1994; Neumann et al., 1995; Bajpai and Brahmi, 1996). Functionally, for example, it elicits Ca^{2+}-independent contraction in smooth muscles and promotes tumor formation in CD-1 mouse skin (Ishihara et al., 1989b; Suganuma et al., 1990). Calyculin A also affects $[Ca^{2+}]_i$ homeostasis. For instance, it enhances L-type Ca^{2+} channel activity (Wiechen et al., 1995). It inhibits Ca^{2+} entry activated by intracellular Ca^{2+} store depletion (capacitative Ca^{2+} entry) in rabbit platelets (Murphy et al., 1996). Recently, we show that calyculin A also abolished capacitative Ca^{2+} entry in HL-60 cells (Leung et al., 1996). These data may suggest a role for PP1 and 2A in capacitative Ca^{2+} entry. In this work, we observed that calyculin A in fact caused a drop in resting $[Ca^{2+}]_i$, and inhibited the spontaneous entry of Mn^{2+} (a surrogate ion for Ca^{2+}). These actions, however, were not exhibited by okadaic acid, a structurally unrelated inhibitor of PP1 and 2A, or by agents which activated protein Ser/Thr kinases. Possible mechanisms of such unique actions of calyculin A will be discussed.

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2. Materials and methods

2.1. Cell culture and drug sources

HL-60 cells obtained from American Type Culture Collection, Maryland, were maintained in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD, USA), 5 μg gentamycin/ml and penicillin/streptomycin (100 U/ml, 100 μg/ml) in a humidified atmosphere with 5% CO_2 at 37°C. The cells were split in a 1:5 ratio every two or three days.

Phorbol myristate acetate (PMA), forskolin and fura 2-AM were obtained from Sigma. Calyculin A and okadaic acid were from Research Biochemicals International (Natick, MA, USA). Staurosporine was purchased from Calbiochem (Richmond, CA, USA).

2.2. Measurement of $[Ca^{2+}]_i$ and divalent cation influx

The methods used to measure $[Ca^{2+}]_i$ and the influx of Mn^{2+} were as described in our previous reports (Leung et al., 1994a,b). Hank's buffered saline solution (HBSS), composed of (mM) NaCl 138, KCl 5.3, $MgSO_4$ 0.8, $CaCl_2$ 1.2, KH_2PO_4 0.44, Na_2HPO_4 0.34, glucose 5, and HEPES (*N*-[2-hydroxyethyl]-piperazine-*N*′-2-ethane sulfonic acid) 25, buffered at pH 7.4, was used for washing the cells. HL-60 cells grown to a density of around 1.5×10^6/ml were harvested, washed once in Ca^{2+}-containing HBSS and resuspended in RPMI 1640 medium at a cell density of about 2×10^7/ml. Fura 2-acetoxymethyl (AM) ester was added to this cell suspension at a final concentration of 5 μM. The cell suspension was then incubated at 37°C for 45 min. Thereafter, the fura 2-loaded cells were washed twice in Ca^{2+}-containing HBSS and resuspended in the same buffer solution at a cell density of 2.8×10^6/ml. Before experimentation of each sample, 1 ml of this cell suspension was centrifuged and the cell pellet was gently resuspended in 2 ml Ca^{2+}-containing or Ca^{2+}-free HBSS. This 2 ml cell suspension was transferred to a quartz cuvette which contained a mini stirrer, and warmed at 37°C for about 5 min before addition of drugs. Fura-2 fluorescence was measured by a Hitachi F-4000 fluorescence spectrophotometer. For $[Ca^{2+}]_i$ measurement, the excitation and emission wavelengths were set at 340 and 500 nm, respectively. F_{max} was obtained by Iysing the cells with 0.1% Triton X-100 in the presence of 1.2 mM Ca^{2+} and F_{min} by chelating Ca^{2+} with 20 mM EGTA. $[Ca^{2+}]_i$ was calculated according to Grynkiewicz et al. (1985). For the measurement of Mn^{2+} influx, the excitation and emission wavelengths were set at 360 and 500 nm, respectively. At 360 nm excitation wavelength, fura-2 fluorescence is insensitive to Ca^{2+} but quenchable by Mn^{2+} (Merritt et al., 1989).

Results are expressed as means ± S.E.M. Student's *t*-test was employed and differences were considered significant when $P < 0.05$.

3. Results

Calyculin A, by its inhibition on PP1 and 2A, has been shown to cause an enhancement of protein Ser/Thr phosphorylation (Murphy and Westwick, 1994; Neumann et al., 1995; Bajpai and Brahmi, 1996). The effects of this drug on $[Ca^{2+}]_i$ homeostasis were investigated in HL-60 cells. Addition of calyculin A (30 nM) caused a decrease in $[Ca^{2+}]_i$ from a basal level of 154 ± 17 to 112 ± 3 nM (Fig. 1A; $P < 0.05$; $n = 5$). The effects of other agents which enhance protein Ser/Thr phosphorylation were also tested. PMA (300 nM), an activator of protein kinase C (PKC), induced a small and variable initial drop in $[Ca^{2+}]_i$ but did not cause any significant changes in $[Ca^{2+}]_i$ during the 6 min after its addition (Fig. 1B). Forskolin (30 μM), which activates the cAMP-protein kinase A (PKA) pathway by means of stimulating adenylate cyclase, raised $[Ca^{2+}]_i$ from 159 ± 15 to 223 ± 16 nM (Fig. 1C; $P < 0.05$; $n = 4$). By contrast to the effect of calyculin A, okadaic acid (1 μM, another inhibitor of PP1 and 2A) raised $[Ca^{2+}]_i$ from 127 ± 8 to 146 ± 10 nM (Fig. 1D; $P < 0.05$, $n = 3$).

We tested whether calyculin A decreased $[Ca^{2+}]_i$ by inhibiting spontaneous Ca^{2+} influx. One approach to examine this is to test whether calyculin A could inhibit the spontaneous entry of Mn^{2+}, a surrogate ion for Ca^{2+}. As

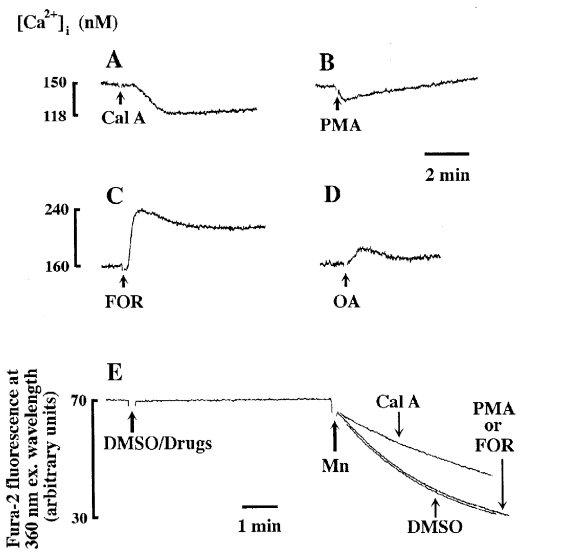

Fig. 1. Effects of calyculin A and other drugs which enhanced protein phosphorylation on $[Ca^{2+}]_i$ and spontaneous Mn^{2+} entry. HL-60 cells in Ca^{2+}-containing HBSS were treated with (A) 30 nM calyculin A, (B) 300 nM PMA, (C) 30 μM forskolin, or (D) 1 μM okadaic acid. In (E), HL-60 cells in Ca^{2+}-free HBSS were treated with DMSO (vehicle control), 30 nM calyculin A, 300 nM PMA or 30 μM forskolin for 5–6 min before the addition of 0.2 mM Mn^{2+}. The *y*-axis represents fura-2 fluorescence at 360 nm excitation wavelength. The traces shown are typical of 3 or more separate experiments.

shown in Fig. 1E, pretreatment with 30 nM calyculin A retarded spontaneous Mn^{2+} entry considerably. The latter was not significantly affected by PMA (300 nM) or forskolin (30 μM). Quantitative results are shown in Fig. 2A. okadaic acid (1 μM) also failed to inhibit Mn^{2+} entry significantly (Fig. 2B). Staurosporine (300 nM), a non-specific inhibitor of protein kinases, did not affect Mn^{2+} entry; neither did it significantly affect the retardation of Mn^{2+} entry by calyculin A (Fig. 2C).

4. Discussion

Calyculin A has been shown to inhibit entry of Ca^{2+} and Mn^{2+} activated by depletion of intracellular Ca^{2+} store (Murphy et al., 1996, Leung et al., 1996). In this work, we observed that calyculin A actually decreased the basal $[Ca^{2+}]_i$ (when cells were bathed in Ca^{2+}-containing medium) and inhibited spontaneous Mn^{2+} entry. These novel actions of calyculin A have not been reported before in any cell type. Calyculin A, at concentrations up to 300 nM, did not affect basal $[Ca^{2+}]_i$ when cells were bathed in Ca^{2+}-free medium (not shown), suggesting that calyculin A did not perturb the intracellular Ca^{2+} store. The lowering effect of calyculin A on basal $[Ca^{2+}]_i$ in Ca^{2+}-containing medium could therefore be due to suppression of spontaneous Ca^{2+} influx or enhancement of the plasmalemmal Ca^{2+} pump. As calyculin A inhibited the entry of Mn^{2+} (a Ca^{2+} surrogate ion which passes through certain plasmalemmal Ca^{2+} channels, but is not a substrate for the plasmalemmal Ca^{2+} pump) (Merritt et al., 1989), it is likely that calyculin A lowered $[Ca^{2+}]_i$ by blocking a plasmalemmal Mn^{2+}-permeable Ca^{2+} leak pathway.

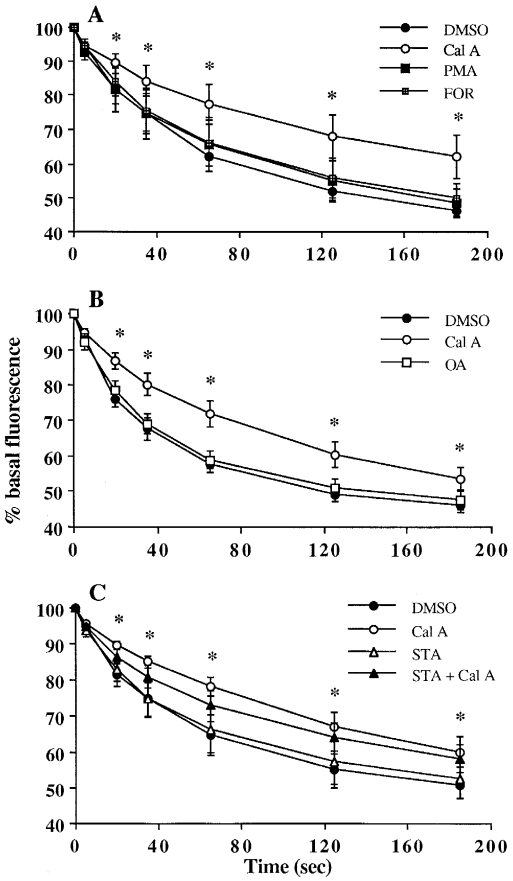

Fig. 2. Quantitative presentation of results obtained from the protocol shown in Fig. 1E. The fluorescence changes after Mn^{2+} addition were quantified in the control (DMSO) and drug-treated groups (treatments with various drugs for 5–6 min did not affect the basal fluorescence before Mn^{2+} addition). The fluorescence values immediately before Mn^{2+} addition were taken as 100% basal fluorescence at time zero. DMSO (solid circle); 30 nM calyculin A (open circle); 300 nM PMA (solid square); 30 μM forskolin (open square with cross); 1 μM okadaic acid (open square); 300 nM staurosporine (open triangle); 300 nM staurosporine plus 30 nM calyculin A (solid triangle). Results are the means ± S.E.M. of 4–7 separate experiments. * Calyculin A-treated groups are significantly ($P < 0.05$) different from DMSO-treated groups.

Calyculin A, a potent and selective inhibitor of PP1 and 2A, is believed to manifest its action by inducing protein Ser/Thr phosphorylation. Thus, calyculin A might lower $[Ca^{2+}]_i$ and suppress Mn^{2+} entry by inhibiting these phosphatases and apparently enhancing protein phosphorylation. Our observations that forskolin and PMA, (activator of PKA and PKC, respectively) did not mimic calyculin A seem to suggest that PKA and PKC were not involved in the actions of calyculin A (also see below). Inhibition of PP1 and 2A by calyculin A and activation of PKA and PKC can phosphorylate different sets of protein substrates (e.g., Bajpai and Brahmi, 1996). Okadaic acid (a PP1 and 2A inhibitor structurally unrelated to calyculin A) was ineffective in decreasing (actually it increased) $[Ca^{2+}]_i$ and very weak in inhibiting Mn^{2+} entry. Calyculin A and okadaic acid have different potencies in inhibiting protein phosphatases: calyculin A inhibits PP1 and PP2A with similar potency (IC_{50} values of 2 and 1 nM, respectively) (Ishihara et al., 1989a), while okadaic acid is considerably less potent in inhibiting PP1 (IC_{50} values from 60 to 500 nM) than PP2A (IC_{50} value of 1 nM) (Hescheler et al., 1988; Ishihara et al., 1989a). Inhibition of PP1 therefore appears to be more important than inhibition of PP2A in mediating calyculin A-induced lowering of $[Ca^{2+}]_i$ and blockade of Mn^{2+} entry. Alternatively, the calyculin A effects could be viewed as resulting from a perturbation of balance between PP1 and PP2A activities, as suggested previously by Obara and Yabu (1993). The differential strengths of calyculin A and okadaic acid in inhibiting PP1 and 2A may result in different protein phosphorylation patterns. It has recently been shown that calyculin A (20 nM), but not okadaic acid (1 μM), caused phosphorylation of the 60 and 78 kDa proteins in YT-INDY natural killer cells (Bajpai and Brahmi, 1996). Therefore, we propose that calyculin A induced a specific pattern of protein phosphorylation, which subsequently led to the blockade of the Mn^{2+}/Ca^{2+} leak pathway. It is also of interest to note that, in guinea-pig intestinal smooth muscle cells, the L-type Ca^{2+} channel current was enhanced when PP1 was

inhibited by high concentrations of okadaic acid and calyculin A, but was suppressed when PP2A was inhibited by low concentrations of these drugs (Obara and Yabu, 1993).

Protein phosphorylation by calyculin A depends on the (basal) activities of protein kinases. Non-specific inhibition of protein kinases (including PKC and PKA) by high concentration of staurosporine did not significantly affect calyculin A-induced inhibition of Mn^{2+} entry. This is consistent with the failure of PMA and forskolin to inhibit influx of Mn^{2+}. Calyculin A-induced retardation of Mn^{2+} entry might therefore involve certain staurosporine insensitive protein kinase(s), the identities of which await future investigation. Some protein Ser/Thr kinases, for instance, casein kinase-1 and calmodulin-dependent protein kinase III, are relatively insensitive to staurosporine (IC_{50} values > 50 μM) (Meggio et al., 1995).

We do not rule out the possibility that calyculin A acted directly on the Ca^{2+}/Mn^{2+} leak pathway. However, Koike et al. (1994) showed that calyculin A did not behave as a Ca^{2+} channel blocker. Whatever the mechanism, it is shown that, amongst the agents which could induce protein Ser/Thr phosphorylation, only calyculin A was able to inhibit the Ca^{2+}/Mn^{2+} leak pathway. The characteristics of such a leak pathway is so far poorly understood, and calyculin A could possibly be a unique tool to probe this pathway.

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