Role of the ERK Pathway in the Activation of Store-mediated Calcium Entry in Human Platelets*  

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Extracellular signal-regulated kinases (ERKs), are common participants in a broad variety of signal transduction pathways. Several studies have demonstrated the presence of ERKs in human platelets and their activation by the physiological agonist thrombin. Here we report the involvement of the ERK cascade in store-mediated Ca\(^{2+}\) entry in human platelets. Treatment of dimethyl-bis(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid-loaded platelets with thapsigargin to deplete the intracellular Ca\(^{2+}\) stores resulted in a time- and concentration-dependent activation of ERK1 and ERK2. Incubation with either U0126 or PD 184352, specific inhibitors of mitogen-activated protein kinase kinase (MEK), prevented thapsigargin-induced ERK activation. Furthermore, U0126 and PD 184352 reduced Ca\(^{2+}\) entry stimulated by thapsigargin or thrombin, in a concentration-dependent manner. The role of ERK in store-mediated Ca\(^{2+}\) entry was found to be independent of phosphatidylinositol 3- and 4-kinases, the tyrosine kinase pathway, and actin polymerization but sensitive to treatment with inhibitors of Ras, suggesting that the ERK pathway might be a downstream effector of Ras in mediating store-mediated Ca\(^{2+}\) entry in human platelets. In addition, we have found that store depletion stimulated ERK activation does not require PKC activity. This study demonstrates for the first time a novel mechanism for regulation of store-mediated Ca\(^{2+}\) entry in human platelets involving the ERK cascade.

In platelets and other nonexcitable cells, stimulation by various agonists results in an increase in the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), which consist of two components: Ca\(^{2+}\) release from internal stores and Ca\(^{2+}\) entry across the plasma membrane (1). The main mechanism for Ca\(^{2+}\) influx is store-mediated Ca\(^{2+}\) entry (SMCE), where the filling state of the internal Ca\(^{2+}\) stores regulates the entry of Ca\(^{2+}\) (2). Although the mechanisms involved in the activation of this pathway are still not well understood, recent studies suggest that a secretion-like coupling model is compatible with the mechanisms underlying SMCR in several cell types (3, 4), including human platelets (5). The secretion-like coupling model proposes a reversible trafficking and coupling of the endoplasmic reticulum with the plasma membrane (3–5). In human platelets we have recently provided the first direct evidence of coupling stimulated by depletion of the intracellular Ca\(^{2+}\) stores, which involves type II liposyn triphosphate receptors in the endoplasmic reticulum and hTRP1 channels in the plasma membrane (6).

As with secretion, the actin cytoskeleton plays a role in the activation (3, 5, 7, 8) and the maintenance of SMCE in platelets (5). However, little is known about the signaling mechanisms involved in the activation of this process. A role for protein tyrosine phosphorylation in the activation of SMCE has been reported in several cell types, including platelets (9–11), where tyrosine phosphorylation is required for actin polymerization (12). In addition, small GTP-binding proteins have been proposed as candidates for the activation of SMCE (13–15). In human platelets, depletion of the Ca\(^{2+}\) stores stimulates translocation and association of Ras with the plasma membrane (16), which is essential for Ras activation (17). In these cells the role of Ras proteins in SMCE is partially mediated by the reorganization of the actin cytoskeleton (16). Activated Ras interacts with several signaling proteins, which include Raf-1, phosphoinositide kinases, diacylglycerol kinase, and MEK (18). Phosphoinositide kinases have recently been shown to modulate actin filament polymerization and SMCE (19). The serine/threonine kinase Raf-1 activates MEK and subsequently the ERK cascade of MAP kinases (18). Since the characterization of ERK1 and ERK2 it has become clear that these proteins are among the protein kinases most commonly activated in signal transduction pathways from cell proliferation to many other events including the production of insulin in pancreatic β cells (20).

We report here that depletion of the intracellular Ca\(^{2+}\) stores evokes Ca\(^{2+}\)-independent activation of ERK kinases and that the ERK kinase pathway is involved in the activation of SMCE in human platelets, probably as a downstream effector of Ras proteins.

**EXPERIMENTAL PROCEDURES**

**Materials—**Fura-2 acetoxymethyl ester (fura-2/AM) was from Texas Fluorescence (Austin, TX). Apyrase (grade VII), aspirin, bovine serum albumin, paraformaldehyde, Nonidet P-40, sodium vanadate, fluorescein isothiocyanate-labeled phalloidin, thrombin, methyl-2,5-dihydroxy-6-cinnamate, and thapsigargin (TG) were from Sigma (Poole, Dorset, United Kingdom). 1,4-Diamino-2,3-diacyclo-1,4-bis-phenylthio)butadiene (U0126), 2-(2-chloro-4-ido-phenylamino)-N-cyclopropylmethoxy-3,4-difluorobenzamide (PD 184352), LY294002, phenylarsine oxide, Ro-31-8220, and cytochalasin D (Cyt D) were from Calbiochem (Nottingham, UK). Farnesylthioacetic acid (FTA) and PP1 were from Alexis Corp. (Nottingham, UK). Phospho-p44/42 ERK monoclonal antibody (E010) was from New England Biolabs (Beverly, MA). Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham Pharmacia Biotech (Little Chalfont, Bucks., UK). Dimethyl-bis(o-aminophenoxy)-ethane-N,N',N'-tetraacetic acid (BAPTA) ace-
toxymethyl ester was from Molecular Probes (Leiden, The Netherlands). All other reagents were of analytical grade.

Platelet Preparation—Fura-2-loaded platelets were prepared as described previously (5). Briefly, blood was obtained from healthy volunteers and mixed with one-sixth volume of acid/citrate dextrorotatory anticoagulant, containing in ml: 85 sodium citrate, 78 citric acid, and 111 n-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 × g and aspirin (100 μM) and apyrase (40 μg/ml) added. Platelet-rich plasma was incubated at 37 °C with 2 μM fura-2/AM for 45 min. For loading with dimethyl-BAPTA, cells were incubated for 30 min at 37 °C with 10 μM dimethyl-BAPTA AM. Cells were then collected by centrifugation at 350 × g for 20 min and resuspended in HEPES-buffered saline containing in (mM): 145 NaCl, 10 HEPES, 10 CaCl2, 5 KCl, 1 MgSO4, pH 7.45, and supplemented with 0.1% (v/v) bovine serum albumin and 40 μg/ml apyrase.

Measurement of Intracellular Free Calcium Concentration ([Ca2+]i)—Fluorescence was recorded from 1.5-ml aliquots of magnetically stirred platelet suspension (101 cells/ml) at 37 °C using a Cairn Research Spectrophotometer (Cairn Research Ltd., Sittingbourne, Kent, UK) with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in [Ca2+]i were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (21).

Determination of [Ca2+]i Entry—Ca2+ influx in platelets that had been store depleted using TG was estimated using the integral of the rise in [Ca2+]i (in arbitrary units) 15% after addition of CaCl2 (5). When platelets were preincubated with inhibitors, Ca2+ entry was corrected by subtraction of the rise in [Ca2+]i, due to leakage of the indicator. TG-induced Ca2+ release was estimated using the integral of the rise in [Ca2+]i, for 3 min after its addition. Thrombin-evoked Ca2+ influx was measured as the integral of the rise in [Ca2+]i, above basal for 1½ min after addition of thrombin in the presence of external Ca2+, corrected by subtraction of the integral over the same period for stimulation in the absence of external Ca2+ (with 100 μM EGTA).

Measurement of F-actin Content—The F-actin content of resting and activated platelets was determined according to a previously published procedure (16). Briefly, washed platelets (2 × 109 cells/ml) were activated in HEPES-buffered saline. Samples of platelet suspension (200 μl) were transferred to 200 μl of ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline for 10 min. Fixed platelets were permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P-40 detergent dissolved in phosphate-buffered saline. Platelets were then incubated for 30 min with fluorescein isothiocyanate-labeled phallolidin (1 μM) in phosphate-buffered saline supplemented with 0.5% (w/v) bovine serum albumin. After incubation the platelets were collected by centrifugation in an MSE Micro-Centaur Centrifuge (MSE Scientific Instruments, Crawley, Sussex, UK) for 60 s at 3000 × g and resuspended in phosphate-buffered saline. Staining of 2 × 109 cells/ml was measured using a PerkinElmer Fluorescence Spectrophotometer (PerkinElmer Life Sciences, Norwalk, CT). Samples were excited at 496 nm and emission was at 516 nm.

Western Blotting—Platelets stimulation was terminated by mixing with an equal volume of 2 × Laemmli’s buffer (22) with 10% dithiothreitol followed by heating for 5 min at 95 °C. One-dimensional SDS electrophoresis was performed with 10% polyacrylamide minigels and separated proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm2, in a semi-dry blottter ( Hoefer Scientific, Newcastle, Staffs., UK) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein-binding sites. Blocked membranes were then incubated with the phospho-p44/42 MAP kinase monoclonal antibody (E10) diluted 1:1500 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 1 min. Blots were then exposed to prefleshed photographic film. Densitometric measurements were made using a Quantimet 500 densitometer (Leica, Milton Keynes, UK).

Statistical Analysis—Analysis of statistical significance was performed using Student’s t test. For multiple comparisons, one-way analysis of variance combined with the Dunnett test was used.

RESULTS

Kinetics and Concentration Dependence of the Ability of Thapsigargin to Activate ERK in Dimethyl-BAPTA-loaded Human Platelets—The activation of ERK was analyzed by Western blotting using a mouse monoclonal phosphospecific anti-

ERK antibody, which only detects the diphosphorylated and so activated form of ERK (23–25). Human platelets were loaded with the Ca2+-chelator dimethyl-BAPTA so as to eliminate Ca2+ but not store depletion-dependent responses. We checked that the rise in [Ca2+]i, evoked by TG, a specific inhibitor of the endomembrane Ca2+-ATPase (sarcoplasmic/endoplasmic reticulum Ca2+-ATPase) (26), was abolished by BAPTA loading (data not shown, but see Ref. 12). Treatment of dimethyl-BAPTA-loaded platelets with 1 μM TG caused rapid activation of both isoforms of ERK (p44 ERK1 and p42 ERK2). As shown in Fig. 1A, ERK activation was detectable 1 min after treatment with TG and reached a maximum within 3 min with an increase of 318.4 ± 12.0% of control. At later times ERK activation decreased much that it was near basal 30 min after TG addition (Fig. 1A, n = 4). The effect of TG on ERK activation was concentration-dependent (Fig. 1B). After treatment of platelets for 3 min with TG a detectable increase in ERK activity was observed at 30 nM, the effect was half-maximal at 151.9 ± 14.3 nm, and maximal at 1 μM (Fig. 1B, n = 4).

U0126 Inhibits TG-induced ERK Activation—U0126 is a cell...
permeant, potent and specific inhibitor of MEK1 and MEK2, the kinases upstream of ERK (27). The effect of U0126 on ERK activation is shown in Fig. 1C. Treatment of dimethyl-BAPTA-loaded human platelets for 30 min at 37°C with U0126 inhibited the TG-induced ERK activation in a concentration-dependent manner, with an IC_{50} of 55.4 ± 1.7 nM and complete inhibition at 10 μM.

Effect of U0126 on TG-evoked SMCE—In a Ca^{2+}-free medium TG evoked a prolonged elevation of [Ca^{2+}], in platelets due to release of Ca^{2+} from the intracellular stores. Subsequent addition of Ca^{2+} (final concentration 300 μM) to the external medium resulted in a sustained increase in [Ca^{2+}], indicative of SMCE (Fig. 1D). Pretreatment of human platelets for 30 min at 37°C with different concentrations of PD184352 (100 nM and 3 μM) decreased TG-evoked Ca^{2+} entry in a concentration-dependent manner (Fig. 1D). U0126-treated cells retained their ability to respond to TG, which indicates that this treatment did not affect the ability of platelets to store Ca^{2+} in intracellular compartments. However, complete inhibition of ERK activation by 10 or 100 μM U0126 reduced Ca^{2+} entry by only 50% (Fig. 1D). These results suggest that SMCE is only partially dependent on ERK activation in human platelets.

Effect of PD 184352 on TG-evoked ERK Activation and SMCE—To further assess the involvement of the ERK cascade in SMCE in human platelets we examined the effect of PD 184352, a selective inhibitor of MEK1 which is structurally unrelated to U0126 (28, 29). Pretreatment of human platelets for 30 min at 37°C with different concentrations of PD 184352 (100 nM and 3 μM) decreased TG-evoked ERK activation in a concentration-dependent manner (data not shown). Treatment with PD184352 inhibits TG-evoked ERK activation by 67% at a concentration of 100 nM while reaching a complete inhibition of ERK activation at 3 μM (n = 4). Consistent with the above, treatment of platelets with PD 184352 reduced TG-induced Ca^{2+} entry in a concentration-dependent manner. Treatment of platelets for 30 min with 100 nM PD 184352 reduced SMCE by 34.1 ± 5.5%. However, as shown for U0126, complete inhibition of TG-evoked ERK activation by 3 μM PD184352 reduced Ca^{2+} entry by only 57% (p < 0.01; n = 6). As with U0126, PD 184352 did not modify the ability of platelets to respond to Ca^{2+} mobilizing agents, such as TG (data not shown).

U0126 Reduces Thrombin-evoked ERK Activation and [Ca^{2+}]_i Elevation—Thrombin is a physiological agonist that stimulates a large number of processes in platelets (1). Previous studies have demonstrated that thrombin induces activation of ERK (30). Here we report that thrombin evokes activation of ERK in dimethyl-BAPTA-loaded platelets, indicating that this response in independent of rises in [Ca^{2+}]. As shown in Fig. 2A, treatment of dimethyl-BAPTA-loaded platelets for 3 min with 1 unit/ml thrombin induced an increase in ERK diphosphorylation of 445.5 ± 27.5% of control (Fig. 2A; n = 3). As shown for TG, preincubation with 10 μM U0126 for 30 min abolished thrombin-induced ERK activation (Fig. 2A; p < 0.001; n = 3)

Treatment of human platelets for 30 min at 37°C with 10 μM U0126 reduced the rise in [Ca^{2+}]), evoked by thrombin (1 unit/ml) in a medium containing 1 mM Ca^{2+} (Fig. 2B). The initial peak [Ca^{2+}]_i elevation above basal after agonist stimulation was significantly reduced from 368.4 ± 45.1 to 207.1 ± 21.1 nM (p < 0.001; n = 6). If we consider the entry of Ca^{2+} stimulated by thrombin (see “Experimental Procedures”), treatment of platelets with 10 μM U0126 significantly reduced thrombin-evoked Ca^{2+} entry by 50.2 ± 3.8 (p < 0.001; n = 6). However, in the absence of external Ca^{2+} (100 μM EGTA added), 10 μM U0126 was without effect on the thrombin-induced rise in [Ca^{2+}]. The initial peak elevation in [Ca^{2+}], above basal after agonist stimulation was 160.8 ± 16.6 nM in control cells and 165.0 ± 22.6 nM in U0126-treated cells (Fig. 2C; n = 6).

Effect of U0126 on the Maintenance of SMCE—To investigate the role of the ERK pathway in the maintenance of SMCE we examined the effect of U0126 on Ca^{2+} entry in platelets after SMCE had been previously stimulated using TG.

Fig. 3 shows the effect of the addition of U0126 to store-depleted human platelets. 10 μM U0126 or the vehicle were added 3 min after TG and cells were then incubated for a further 30 min before the addition of Ca^{2+} to the medium (final concentration 300 μM) to initiate Ca^{2+} entry. As shown in Fig. 3, at the time when U0126 was added Ca^{2+} entry was already stimulated (Control t = 3 min). Addition of U0126 after activation of SMCE did not significantly alter Ca^{2+} entry (Fig. 6; p = 0.77; n = 6). These observations suggest a role for the ERK cascade in the activation but not in the maintenance of SMCE. In addition, these findings demonstrate that U0126 does not act either as a Ca^{2+} channel blocker or a Ca^{2+} chelator.

Role of ERK Pathway in TG-induced Actin Polymerization in Human Platelets—A role for the actin cytoskeleton in SMCE...
has been suggested in several cell types (7, 31). We have previously shown that TG induces actin polymerization in dimethyl-BAPTA-loaded human platelets (16). Stimulation of dimethyl-BAPTA-loaded platelets with 1 μM TG in a Ca²⁺-free medium raised F-actin content by 34.1 ± 2.9% compared with control unstimulated cells. Treatment of human platelets with U0126 at concentrations of 100 nM or 10 μM did not significantly alter the F-actin content of resting or TG-treated platelets (Table I; p = 0.80; n = 6) suggesting that the role of ERK in SMCE is not mediated via actin polymerization. Similar results were obtained when PD 184352 was used. Treatment of platelets with 100 nM or 3 μM PD 184352 did not modify the F-actin content either in unstimulated or in TG-treated cells (data not shown). Consistent with the results reported above, treatment of human platelets for 30 min with 100 nM or 10 μM U0126 after SMCE had been activated by the addition of TG did not significantly modify TG-evoked increase in the F-actin content (Table I; p = 0.82; n = 6).

Since depolarization of the membrane potential has been reported to reduce the driving force for agonist- and store-depletion-evoked Ca²⁺ entry (32), we have investigated whether the effect of U0126 could be attributed to changes in membrane potential. We studied the effects of this inhibitor on SMCE in the presence of the K⁺-ionophore valinomycin, which stabilizes the platelet membrane potential close to the K⁺-equilibrium potential (33). Treatment of platelets with 10 μM U0126 inhibited SMCE to the same extent in the presence or absence of valinomycin (3 μM; data not shown). This finding indicates that the effect of U0126 is not due to a reduction in the membrane potential and in agreement with previous studies (27, 29) indicates that the inhibition of SMCE by U0126 is likely to be explained by blockade of the ERK cascade in human platelets.

Involvement of the ERK Cascade in the Secretion-like Coupling Model—As reported previously, secretion-like coupling is the model that best describes the mechanism of activation of SMCE in several cell types (3, 4), including platelets (5, 6). A number of studies have suggested a role for Ras proteins in the activation of SMCE (13–16). We have recently reported that depletion of the intracellular stores using TG in dimethyl-BAPTA-loaded platelets induced translocation and association of Ras with the plasma membrane, which has been shown to be essential for its activation (17). This process was prevented by treatment with the farnesylcysteine analog, FTA (16). In these cells, Ras activity is required for both the activation of SMCE, by a mechanism partially dependent on the actin cytoskeleton, and its maintenance (16). To investigate whether the ERK cascade is a downstream effector of Ras in the activation of SMCE, we examined the effect of FTA on TG-induced ERK activation. As shown in Fig. 4A, treatment of dimethyl-BAPTA-loaded platelets with 40 μM FTA for 10 min had no effect on basal ERK activity but significantly reduced TG-induced ERK activation. FTA treatment reduced TG-induced ERK activation to 7.1 ± 4.3% of control (p < 0.001; n = 4). In contrast, treatment of human platelets for 40 min with 10 μM Cyt D, conditions that prevent actin polymerization in these cells (5, 16, 34), did not modify either basal or TG-stimulated ERK activation. These findings suggest that Ras is an upstream regulator of the ERK cascade, an effect which might belong to the actin cytoskeleton-independent branch of the Ras-dependent activation of SMCE in these cells.

We further investigated if other components of the secretion-like coupling model, such as the phosphatidylinositol 3- and 4-kinases (PI3K and PI4K; Ref. 19) or protein tyrosine kinases (12), modulate the activity of ERK. As shown in Fig. 4B, incubation of human platelets at 37 °C in the presence of either 1 μg/ml methyl-2,5-dihydroxycinnamate for 30 min, a treatment that we have recently show to abolish store depletion-induced tyrosine phosphorylation (12), or 10 μM PP1 for 10 min, to inhibit the tyrosine kinases of the Src family, had no effect on either basal or store depletion-induced ERK activation (n = 3). Furthermore, we have found that inhibition of protein-tyrosine phosphatases by treatment of platelets for 30 min with 10 μM phenylarsine oxide or 100 μM sodium vanadate had no effect on U0126-induced inhibition of SMCE (Fig. 4C; n = 4), providing further evidence for the independence of the ERK cascade from the protein tyrosine phosphorylation/dephosphorylation process. Preincubation of platelets with 100 μM LY294002 for 30 min has been shown to inhibit both PI3K and PI4K in human platelets (19). Treatment of platelets with 100 μM LY294002 did not alter basal or store depletion-stimulated ERK activation (Fig. 4B, n = 3). Since both tyrosine kinases and phos-
phosphatidylinositol kinases have been shown to be involved in the cytoskeleton-dependent branch of Ras-dependent activation of SMCE (12, 19), these findings further suggest that the ERK cascade might be a component of the actin cytoskeleton-independent pathway of Ras-mediated SMCE in platelets.

Protein kinase C (PKC), has been shown to be important in mediating G_q-dependent activation of the ERK cascade (18). To investigate whether PKC activity is required for store depletion-induced ERK activation, we examined the effect of the PKC inhibitor Ro-31-8220. Treatment of human platelets for 5 min with Ro-31-8220 (3 μM) was without effect on either basal or store depletion-induced ERK activation (Fig. 4B; n = 3), indicating that store depletion-stimulated ERK activation does not require PKC activity.

**Complementary Effects of ERK and the Actin Cytoskeleton in Ras-dependent SMCE in Human Platelets**—To further investigate whether the ERK cascade and actin polymerization are two independent pathways involved in Ras-dependent activation of SMCE, we have investigated the combined effect of both mechanisms on the activation of SMCE. Human platelets were incubated at 37 °C in the absence or presence of 10 μM PAO, for 10 min or 10 μM Cyt D for 40 min as indicated and then treated with 1 μM TG for 3 min and lysed. B, dimethyl-BAPTA-loaded human platelets were preincubated at 37 °C in the absence or presence of 1 μM methyl-2,5-dihydroxycinnamate (M-2,5-DHC) for 30 min, 10 μM PP1 for 10 min, 100 μM LY294002 for 30 min, or 3 μM Ro-31-8220 for 5 min as indicated and then stimulated with 1 μM TG for 3 min and lysed. Samples were subjected to SDS-PAGE and analyzed by Western blotting with the specific phospho-p44/42 ERK monoclonal antibody (E10) as described under “Experimental Procedures.” Bands were revealed using chemiluminescence. The panels show results from one experiment representative of three others. C, fura-2-loaded human platelets were incubated at 37 °C for 30 min in the presence of 1 μM phenylarsine oxide (PAO), 100 μM sodium vanadate or the vehicle and then with U0126 (10 μM) or the vehicle (Control) for a further 30 min. Cells were then stimulated with TG (200 nM) in a Ca^2+-free medium. Three min later CaCl_2 (final concentration 300 μM) was added to the medium to initiate Ca^2+ entry. Elevations in [Ca^2+]_i were monitored using the 340/380 nm ratio and traces were calibrated in terms of [Ca^2+]_i. Traces shown are representative of four separate experiments.

DISCUSSION

ERKs are thought to act as a point of convergence of multiple cellular signaling pathways since they are activated by a broad range of biochemical signals. The ERK cascade contains at least three protein kinases that work in series. The first of these three kinases is a Raf isoform, commonly Raf-1, which is usually activated by Ras. Once activated Raf-1 phosphorylates MEKs, a family of dual-specificity protein kinases that phosphorylate two residues, a threonine and a tyrosine, to activate their ERK targets (18, 35).

Blood platelets, which are anucleate nondifferentiating cells with no growth potential, are a useful model for studying the involvement of ERK in cellular signaling, independently of nuclear DNA-dependent pathways. Two forms of ERK have been identified in human platelets, p44 ERK1 and p42 ERK2, stimulated by thrombin, collagen, or phorbol esters (36, 37). However, the ERK signal pathway in platelets remains largely uncharacterized.

The results presented here demonstrate that depletion of the intracellular Ca^2+ stores using TG induced activation of ERK1...
and ERK2, a process which, to our knowledge, is for the first time here shown to be independent of rises in \([\text{Ca}^{2+}]\). In this respect, our data differ from those reported by Chao et al. (38) in human foreskin fibroblast (HSWP) cells and A431 cells. In that study, TG-induced ERK activation was \(\text{Ca}^{2+}\)-dependent; however, epidermal growth factor increased ERK activity was found to be independent of \([\text{Ca}^{2+}]\) elevation. This may reflect the fact that the ERK cascade is regulated by different mechanisms in different cell types, although the discrepancy may also be due to differences in the experimental protocol. Chao et al. (38) studied the activation of ERK after a 15-min treatment with EGTA, which was shown to fully deplete the intracellular \(\text{Ca}^{2+}\) stores, while we have found that in platelets ERK activation declines significantly 3 min after depletion of the \(\text{Ca}^{2+}\) stores has commenced.

The development in recent years of inhibitors of the ERK cascade, such as U0126 and PD 184352, two potent and highly specific inhibitors of MEK (27–29), have been useful for identifying the cellular functions of the ERK pathway. Indeed, both U0126 and PD 184352 inhibited ERK activation stimulated by depletion of the \(\text{Ca}^{2+}\) stores using TG. To evaluate the possible involvement of the ERK pathway in SMCE we examined the effect of these inhibitors on TG-evoked SMCE. Our results demonstrate that both agents reduced SMCE in a concentration-dependent manner. However, we found that complete inhibition of ERK activation reduced \(\text{Ca}^{2+}\) entry only by 50%, suggesting that the ERK cascade is only partially required for the activation of SMCE. The finding that U0126 and PD 184352 inhibit \(\text{Ca}^{2+}\) entry was confirmed with the use of thrombin, a physiological agonist that stimulates a large number of processes in human platelets, including calcium mobilization and ERK activation (1, 36, 37). In addition, the present study shows that thrombin-induced ERK activation does not require rises in \([\text{Ca}^{2+}]\). Our results clearly show that U0126 inhibited thrombin-induced \(\text{Ca}^{2+}\) elevation in the presence of 1 mM external \(\text{Ca}^{2+}\) without having any effect on the release of \(\text{Ca}^{2+}\) from the intracellular stores. In agreement with the observations reported above, these findings suggest that the role of ERK on thrombin-induced \(\text{Ca}^{2+}\) mobilization occurs entirely through the modulation of \(\text{Ca}^{2+}\) entry.

It remains to be elucidated how the ERK pathway acts to mediate SMCE. The actin cytoskeleton has been proposed to play a key role in the activation of SMCE in several cell types (3, 7, 8), including human platelets (31). Hence, we have investigated the effect of the ERK cascade inhibitors, U0126 and PD 184352, on store depletion-induced actin polymerization. Our results clearly show that neither U0126 nor PD 184352 modify TG-induced actin polymerization in human platelets. These observations indicate that ERK-mediated activation of SMCE is unlikely to occur as a result of modulation of actin reorganization.

We have previously reported that different mechanisms are required for the activation and maintenance of SMCE in platelets (12, 31). Since the ERK cascade participates in the activation of SMCE we also investigated its possible role in the maintenance of the process. Addition of the MEK inhibitor U0126 after the activation of SMCE did not reverse either actin polymerization or \(\text{Ca}^{2+}\) entry activated by TG. This suggests that the ERK cascade is not required for the maintenance of SMCE. The lack of effect of ERK on the maintenance of SMCE and actin reorganization is consistent with previous studies reporting that the actin cytoskeleton is essential for the maintenance of the secretion-like coupling process underlying SMCE (5).

The results presented above indicate that the effect of U0126 on \(\text{Ca}^{2+}\) entry is not likely to be mediated by nonspecific effects as a \(\text{Ca}^{2+}\) chelator or \(\text{Ca}^{2+}\) channel blocker, since it did not have any effect on \(\text{Ca}^{2+}\) entry when added after depletion of the stores. In addition, our results indicate that these inhibitors are effective at selectively inhibiting \(\text{Ca}^{2+}\) entry, as demonstrated by the lack of effect on \(\text{Ca}^{2+}\) storage or agonist-evoked \(\text{Ca}^{2+}\) release. Depolarization of the membrane potential has been shown to decrease the driving force for \(\text{Ca}^{2+}\) entry (32). To address this issue we have conducted experiments in the presence of valinomycin, a potassium ionophore that clamps the platelet membrane potential close to the potassium equilibrium potential (33). Our results indicate that U0126 inhibited \(\text{Ca}^{2+}\) entry to the same extent in the absence or presence of valinomycin. Furthermore, U0126 was effective at reducing the activation but not the maintenance of SMCE, hence, the effect of this inhibitor cannot be attributable to a modification of membrane potential. Therefore, these results together with others presented in this paper indicate that the effect of U0126 on SMCE is more likely to be explained by inhibition of the ERK pathway. These observations are in agreement with previous studies reporting the high specificity of U0126 and PD 184352 as inhibitors of MEK over many other protein kinases (29).

A role for the Ras family of small GTPases in SMCE has been suggested in several cell types, including platelets (13–16). In human platelets we have recently reported that the involvement of Ras in SMCE is mediated via two different, actin cytoskeleton-dependent and cytoskeleton-independent pathways (16). Ras is commonly required for the activation of Raf-1, the first kinase of the ERK cascade (39). Hence, the possibility that Ras could mediate TG-induced ERK activation was investigated. We conducted a series of experiments wherein platelets were incubated with the farnesylcysteine analog FTA at a concentration that we have previously reported to inhibit membrane association and thus activation of Ras (16). Treatment of platelets with FTA caused no changes in basal ERK activity but abolished TG-induced ERK activation. In contrast, no effect was observed after treatment of platelets for 40 min with 10 \(\mu\text{M}\) Cyt D, which completely inhibits TG-induced actin polymerization (16). Thus, TG-induced ERK activation is likely to be mediated by Ras but is independent of the actin cytoskeleton. Therefore, the ERK cascade is a possible downstream effector of Ras that might be involved in the cytoskeleton-independent branch of the Ras-mediated SMCE mechanism. Consistent with the above, we have found that the activity of phosphatidylinositol 3- and 4-kinases or tyrosine kinases, which modulate SMCE through the reorganization of the actin cytoskeleton (12, 19), is not required for store depletion-induced ERK activation in platelets. Furthermore, inhibition of protein-tyrosine phosphatases did not impair U0126-induced inhibition of SMCE. These findings provide further evidence for the involvement of the ERK cascade on the actin cytoskeleton-independent branch of the Ras-dependent activation of SMCE in human platelets.

In agreement with the above, we have previously reported (16) that treatment with FTA resulted in a massive reduction in SMCE while Cyt D or U0126 were about half as effective as FTA. Furthermore, the effects of Cyt D and U0126 were found to be additive. This indicates that the actin cytoskeleton and the ERK cascade mediate SMCE by independent pathways. Our results allow us to propose that the ERK pathway might be a candidate for the cytoskeleton-independent branch of Ras-mediated SMCE in human platelets.

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J. Biol. Chem. 2001, 276:15659-15665. doi: 10.1074/jbc.M009218200 originally published online January 26, 2001

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