Effect of temperature on bradykinin-induced arachidonate release and calcium mobilization in vascular endothelial cells

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The effect of decreased temperature on Ca^{2+} -dependent arachidonic acid release was studied in vascular endothelial cells by investigating bradykinin (BK)-stimulated Ca^{2+} mobilization, inositol phosphate formation and arachidonic acid release. At both 37 °C and 22 °C, BK efficiently increased cytosolic Ca^{2+} concn. ($[\text{Ca}^{2+}]_i$). At 22 °C, peak $[\text{Ca}^{2+}]_i$ was higher and returned to basal levels more slowly. Although this response was preceded by rapid formation of $\text{Ins}(1,4,5)\text{P}_3$, the activity of phospholipase C was significantly impaired at 22 °C. To determine if $\text{Ins}(1,4,5)\text{P}_3$ effectively mobilized intracellular Ca^{2+} , we used saponin-permeabilized cells. $\text{Ins}(1,4,5)\text{P}_3$ mobilized sequestered Ca^{2+} to a similar degree at 37 °C and 22 °C, although Ca^{2+} release was prolonged at 22 °C. In intact cells, BK mobilized intracellular Ca^{2+} stores and activated Ca^{2+} entry. The rate of $^{45}\text{Ca}^{2+}$ entry was approx. 2-fold slower at 22 °C, even though the peak and

duration of the rise in $[\text{Ca}^{2+}]_i$ were higher and sustained at the lower temperature. TG mobilized intracellular Ca^{2+} , activated Ca^{2+} entry and elevated $[\text{Ca}^{2+}]_i$ at both temperatures. As with BK, the peak $[\text{Ca}^{2+}]_i$ reached after thapsigargin treatment was higher at 22 °C. This effect of lower temperature on $[\text{Ca}^{2+}]_i$ was most probably due to decreased Ca^{2+} efflux after a decrease in activity of the Ca^{2+} -ATPase on the plasma membrane. Both A23187 and BK were shown to stimulate phospholipase A_2 and arachidonic acid release at 22 °C. In each case, the rate and extent of release were decreased compared with that at 37 °C. Among several effects, lowering the temperature decreases the activity of phospholipase C, Ca^{2+} -ATPase(s), Ca^{2+} -entry mechanisms and phospholipase A_2 . Together, these effects lead to a higher and more prolonged elevation of $[\text{Ca}^{2+}]_i$, but a decrease in arachidonate release in response to BK.

INTRODUCTION

Prostacyclin is synthesized from unesterified arachidonic acid by vascular endothelial cells and participates in the regulation of vascular tone, platelet aggregation and neutrophil function. Agonists which stimulate prostacyclin production do so by increasing the release of arachidonate from membrane phospholipids following activation of phospholipase A_2 [1,2]. Mechanisms by which phospholipase A_2 is regulated are not completely understood.

Both BK and ATP potentially stimulate arachidonic acid release and prostacyclin formation in endothelial cells [2–4]. These agonists bind to specific receptors, activate phospholipase C and initiate hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$, producing $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol [4,5]. $\text{Ins}(1,4,5)\text{P}_3$ mobilizes intracellular Ca^{2+} from the endoplasmic reticulum, and diacylglycerol activates protein kinase C. At the same time an influx mechanism is activated which allows extracellular Ca^{2+} to enter the cell [6–8]. Release of intracellular Ca^{2+} stores and entry of Ca^{2+} from outside is thought to participate in the full activation of phospholipase A_2 and arachidonate release [9].

Although numerous studies have shown that Ca^{2+} is important for activation of phospholipase A_2 and arachidonic acid release [1–5,9], others have shown a clear dissociation [10,11]. For example, in thyroid cells it has been reported that noradrenaline stimulates arachidonate release by acting at receptors directly coupled to phospholipase A_2 such that activation is independent of inositol phosphate formation and elevation of cytosolic Ca^{2+} concn. ($[\text{Ca}^{2+}]_i$) [10]. It has also been reported that activation of protein kinase C is important for regulating phospholipase A_2 [10–13]. Presumably phosphorylation of phospholipase A_2 lowers its K_m for Ca^{2+} so that it is fully active at basal Ca^{2+} levels [12,13].

In our own studies in endothelial cells, we have reported both an AlF_4^- -activated Ca^{2+} -independent pathway and a BK-activated Ca^{2+} -dependent pathway for phospholipase A_2 activation [9]. In contrast, others have used lower temperature (30 °C rather than 37 °C) to demonstrate that BK can activate phospholipase A_2 and arachidonic acid release with no apparent activation of phospholipase C [14]. In those studies no measurable $\text{Ins}(1,4,5)\text{P}_3$ was found at the lower temperature, implying that there was no change in $[\text{Ca}^{2+}]_i$, and thus the mechanism for arachidonic acid release was Ca^{2+} -independent. However, the effect of temperature on Ca^{2+} mobilization was not investigated.

Failure of BK to activate phospholipase C and mobilize Ca^{2+} in endothelial cells at lower temperatures is somewhat surprising. For example, it has been reported that $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} release from the endoplasmic reticulum is temperature-insensitive [15,16]. Furthermore, many studies have been done measuring agonist-induced Ca^{2+} currents at room temperature. Recent studies using avian nasal-gland cells showed that lowering the temperature from 38 °C to 27 °C did not prevent mobilization of intracellular Ca^{2+} by muscarinic agonists, but instead promoted a higher and more prolonged elevation of $[\text{Ca}^{2+}]_i$ [17]. Although $\text{Ins}(1,4,5)\text{P}_3$ levels were not determined, it is unlikely that phospholipase C activity was completely inhibited at 27 °C. In fact, Ca^{2+} mobilization from intracellular stores occurred at lower temperatures. The primary effect of temperature on Ca^{2+} mobilization appeared to be on Ca^{2+} efflux, leading to the suggestion that Ca^{2+} -ATPases are inhibited, allowing $[\text{Ca}^{2+}]_i$ to reach higher, more persistent, levels [17]. If mammalian endothelial cells are similarly affected by lower temperature, one might predict that Ca^{2+} -dependent processes such as activation of phospholipase A_2 would be activated in these cells in response to the rise in $[\text{Ca}^{2+}]_i$. However, the effects of temperature on cell

Abbreviations used: BK, bradykinin; TG, thapsigargin; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; HBSH, Hanks' balanced salt solution containing 0.25 % BSA and 10 mM Hepes, pH 7.4.

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signalling mechanisms have not been carefully studied in mammalian cells.

The purpose of the present study was to investigate the effects of temperature on BK-induced arachidonic acid release in endothelial cells. We determined the effect of temperature on several steps in the signalling pathway, including inositol phosphate metabolism, Ca^{2+} mobilization from intracellular sites, Ca^{2+} entry and arachidonic acid release. BK was found to activate phospholipase C, to mobilize intracellular Ca^{2+} and to activate phospholipase A_2 at both 22 °C and 37 °C. Although the elevation of $[\text{Ca}^{2+}]_i$ in response to BK was higher at 22 °C, arachidonic acid release was found to occur less efficiently at the lower temperature.

EXPERIMENTAL

Materials

BK triacetate, Cytodex 3 beads and collagenase type 1 were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); fura-2 AM (acetoxymethyl ester) was from Behring Diagnostics (San Diego, CA, U.S.A.); $^{45}\text{CaCl}_2$, *myo*-[2- ^3H]inositol and [5,6,8,9,11,12,14,15- ^3H]arachidonic acid were from New England Nuclear (Wilmington, DE, U.S.A.); thapsigargin (TG) was from LC Services (Woburn, MA, U.S.A.); Dulbecco's modified Eagle's medium, Hanks' balanced salt solution, antibiotic/antimycotic (10000 units of penicillin, 10000 units of streptomycin and 25 μg of fungizone/ml), and trypsin (0.5 g/l)/EDTA (0.2 g/l) were from Grand Island Biological Co. (Grand Island, NY, U.S.A.); fetal-calf serum was from Hyclone (Logan, UT, U.S.A.); anion-exchange resin AG1X8 (100–200 mesh) was from Bio-Rad (Richmond, CA, U.S.A.); and tissue-culture plasticware was from NUNC (PGC Scientific Corp., Gaithersburg, MD, U.S.A.).

Cell culture

Endothelial cells were isolated and grown as previously described [2,5,8]. They exhibited the typical cobblestone appearance and expressed Factor VIII-related antigen. Cells were passaged after brief exposure to trypsin/EDTA and seeded into flasks or directly on to 1.25 cm $\times$ 3 cm glass coverslips (30000 cells/coverslip). Cells in passages 3–8 were used in these studies. Cultures of endothelial cells on Cytodex 3 beads were established as previously described [9].

Measurement of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was monitored continuously as previously described [8,9]. Briefly, monolayers of cells were grown to confluence on glass coverslips and incubated for 45 min at 37 °C with 20 μM fura-2 AM in Hanks' balanced salt solution containing 0.25 % BSA and 10 mM Hepes (HBSH), pH 7.4. The coverslips were placed in a 3 ml flow-through cuvette. Buffer flow was maintained at 3 ml/min and the temperature regulated. Rapid exchange of cuvette contents was accomplished by injecting 20 ml of an appropriate buffer or agonist solution into the cuvette. BK was added to the cuvette over 1 min and washed out after 2 min. $[\text{Ca}^{2+}]_i$ was calculated as previously described [8,9,18]. The K_d used to calculate $[\text{Ca}^{2+}]_i$ was corrected for temperature as described in [17].

Ca^{2+} uptake

To measure Ca^{2+} entry, confluent monolayers of endothelial cells in six-well plates were incubated in 0.75 ml of buffer containing 3 μCi of $^{45}\text{Ca}^{2+}$. Ca^{2+} uptake was initiated by addition of 1 μM

BK. Incubations were stopped by quickly removing the buffer and washing cells three times with ice-cold buffer. Cells were disrupted and Ca^{2+} was released by adding 0.5 ml of 6 % trichloroacetic acid. A sample of the trichloroacetic acid extract was used to determine the accumulation of $^{45}\text{Ca}^{2+}$.

Inositol polyphosphate production

Production of inositol polyphosphates in response to BK was determined by using modifications of previously published procedures [18]. Cells were seeded into six-well plates (9 cm²/well), grown to confluence and incubated for 24 h in 1.5 ml of medium containing 10 μCi of [^3H]inositol/ml. Labelled cells were washed twice with HBSH containing 10 mM LiCl (pH 7.4) and preincubated with the same buffer for 10 min. Agents to be tested were added and the reaction was stopped at various times, and labelled inositol phosphates were extracted and analysed by anion-exchange chromatography using AG1X8 (100–200 mesh) anion-exchange resin [18]. Inositol monophosphates (InsP_1), inositol bisphosphates (InsP_2) and inositol trisphosphates (InsP_3) were respectively eluted with 8 ml each of 0.2 M, 0.4 M and 1.0 M ammonium formate in 0.1 M formic acid. Retention times were verified by using labelled standards.

$\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release

The effect of temperature on $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release from intracellular stores was studied by using saponin-permeabilized endothelial cells. Cells at confluence were treated with trypsin, washed and suspended at 5×10^6 cells/ml in permeabilization buffer containing 100 mM KCl, 40 mM Tris/HCl, 1 mM KH_2PO_4 , 10 mM NaCl, 4 mM MgCl_2 , 10 μM antimycin A, 10 μg /ml oligomycin, 1 μg /ml leupeptin and 0.1 mg/ml soybean trypsin inhibitor. The cell suspension (1 ml) was added to a stirred cuvette and buffer Ca^{2+} concentration was monitored as described above. Then 1 μM fura-2 (free acid), 24 μg of saponin/ 10^6 cells, 10 mM phosphocreatine and 2 units of creatine kinase/ml were added. The saponin concentration used permeabilized 85–98 % of all cells, as determined by Trypan Blue staining. Starting Ca^{2+} concentrations were adjusted to approx. 1 μM by using CaCl_2 , and 3 mM ATP (potassium salt, vanadate-free) was added to initiate Ca^{2+} sequestration into intracellular stores. Under these conditions, ATP addition typically resulted in a net uptake of 180 pmol of Ca^{2+} / 10^6 cells and the cells responded to multiple doses of $\text{Ins}(1,4,5)\text{P}_3$ without desensitization.

Fatty acid release

To measure arachidonic acid release, confluent monolayers of endothelial cells on beads or T-80 cm² flasks were labelled by incubation overnight with 20 μCi of [^3H]arachidonate/ 2×10^7 cells. Cells on beads were loaded into columns (1.0 cm diameter, 1.5 cm height, 1.0 ml total volume; 2×10^6 cells/column) and perfused at 1 ml/min with HBSH containing 0.25 % BSA (fatty acid-free), pH 7.4. Unincorporated label was removed by washing for 20 min. The experiment was begun, and a 1 ml fraction was collected every 1 min and analysed for radioactivity. Cells in flasks were washed, fresh buffer was added and samples were taken to determine arachidonic acid release.

Statistical analysis

Data were analysed by analysis of variance. Multiple comparisons were made with either Newman-Keuls' or

Dunnett's *post hoc* tests. Data are given throughout as means $\pm$ S.E.M.

RESULTS

We began to investigate the effects of temperature on the response of endothelial cells to BK by monitoring changes in $[\text{Ca}^{2+}]_i$ in fura-2-loaded cells perfused with buffer held at either 37 °C or 22 °C. At 37 °C, BK (1 μM) initiated a rapid elevation of $[\text{Ca}^{2+}]_i$. $[\text{Ca}^{2+}]_i$ levels rose to 756 ± 60 nM by 0.34 min and returned quickly to baseline (Figure 1). At 22 °C, $[\text{Ca}^{2+}]_i$ rapidly reached a higher peak value (887 ± 60 nM) by 0.68 min and returned to

Figure 1 Effect of temperature on BK-induced Ca^{2+} mobilization in endothelial cells

Confluent monolayers of fura-2-loaded endothelial cells on coverslips were perfused with buffer at either 37 °C or 22 °C. BK (1 μM) was added as indicated to initiate Ca^{2+} mobilization. Data presented as means $\pm$ S.E.M. ($n = 4$). Peak $[\text{Ca}^{2+}]_i$ reached at 22 °C was significantly higher than that reached at 37 °C ($P < 0.05$).

baseline more slowly. The baseline after BK treatment was unaffected by the temperature and was indistinguishable from the initial baseline. Clearly, BK was able to elevate $[\text{Ca}^{2+}]_i$ efficiently in cells maintained at lower temperatures. However, the delayed peak value and the slower return to basal $[\text{Ca}^{2+}]_i$ suggested kinetic differences in certain aspects of the response.

Mobilization of Ca^{2+} occurs in response to phospholipase C activation and formation of inositol phosphates. In the following experiments, the effect of temperature on inositol phosphate formation was studied in cells prelabelled with [^3H]inositol at 37 °C and stimulated with BK at either 37 °C or 22 °C. Total labelled inositol phosphates were extracted and individual isomers isolated by ion-exchange chromatography. Although significant amounts of inositol phosphates were formed at lower temperature, both total labelled inositol phosphates (sum of the isomers) and total InsP_3 (area under the curve) were decreased at 22 °C (Figure 2). Thus phospholipase C is activated at 22 °C by BK, although its activity is significantly lower than at 37 °C.

Elevation of $[\text{Ca}^{2+}]_i$ in endothelial cells reflects the combined activities of agonist-induced Ca^{2+} release from intracellular stores and activation of Ca^{2+} entry from outside the cell [6–8]. To determine directly the effect of temperature on mobilization of Ca^{2+} from $\text{Ins}(1,4,5)\text{P}_3$ -responsive intracellular stores, saponin-permeabilized endothelial cells were used. At 37 °C addition of ATP to permeabilized cells led to a rapid sequestration of Ca^{2+} into intracellular stores (171 ± 13 pmol/ 10^6 cells) (Figure 3). The rate of Ca^{2+} uptake was somewhat slower at 22 °C, although the same amount of Ca^{2+} was sequestered (172 ± 30 pmol/ 10^6 cells). Addition of $\text{Ins}(1,4,5)\text{P}_3$ at 37 °C or 22 °C led to rapid release of approx. 70% and 79% respectively of the stored Ca^{2+} . At 37 °C the response to $\text{Ins}(1,4,5)\text{P}_3$ was transient, probably reflecting the combined activities of inositol phosphatases and the Ca^{2+} -ATPase on the endoplasmic reticulum rather than desensitization of the $\text{Ins}(1,4,5)\text{P}_3$ receptor, since the preparation responded equally well to a second challenge with $\text{Ins}(1,4,5)\text{P}_3$ (results not shown). At 22 °C the response to $\text{Ins}(1,4,5)\text{P}_3$ was prolonged. Thus, $\text{Ins}(1,4,5)\text{P}_3$ mobilizes intracellular Ca^{2+} efficiently at lower temperatures.

Data in Figures 2 and 3 suggest that BK mobilizes intracellular Ca^{2+} in intact cells at 22 °C. In the following experiments, we investigated the effects of temperature on mobilization of intracellular Ca^{2+} stores, using intact cells under conditions where

Figure 2 Effect of temperature on BK-induced inositol phosphate formation

[^3H]inositol-labelled cells were stimulated with 1 μM BK, and labelled inositol phosphates were analysed by anion-exchange chromatography. At each time point, radioactivity from unstimulated cells was subtracted from that recovered from stimulated cells, and the net increase is reported. Data are given as means $\pm$ S.E.M. ($n = 3$). Incubation at 22 °C significantly inhibited [^3H]InsP₁ and [^3H]InsP₂ formation at each time point ($P < 0.01$).

Figure 3 Effect of temperature on Ins(1,4,5) P_3 -induced Ca^{2+} mobilization

Cells were suspended in permeabilization buffer containing 1 μ M fura-2 and permeabilized with saponin. An ATP-generating system and 3 mM ATP were added to initiate Ca^{2+} sequestration into intracellular sites. Ins(1,4,5) P_3 (1 μ M) was added to induce Ca^{2+} release from storage sites. Data are given as means $\pm$ S.E.M. ($n = 4$).

we dissociated Ca^{2+} release from intracellular sites and Ca^{2+} entry from outside the cell. We have recently shown that mobilization of the intracellular Ca^{2+} pool leads to activation of Ca^{2+} entry, which continues until the pool is refilled [8]. In these studies, addition of BK to cells perfused with Ca^{2+} -free HBSH containing 1 mM EGTA led to Ca^{2+} release from intracellular stores, whereas subsequent perfusion with Ca^{2+} -complete HBSH (1.4 mM Ca^{2+}) led to Ca^{2+} entry which continued until the intracellular pool was refilled [8]. Using this approach in the

Figure 5 Effect of temperature on BK-induced $^{45}Ca^{2+}$ entry

Cells incubated at either 37 °C or 22 °C and 1 μ M BK added to initiate $^{45}Ca^{2+}$ uptake. Ca^{2+} entry was determined by measuring the extent of $^{45}Ca^{2+}$ accumulation after rinsing the cells. Data given as means $\pm$ S.E.M. ($n = 6$). $^{45}Ca^{2+}$ influx at 22 °C was significantly decreased as compared with that at 37 °C ($P < 0.01$).

present studies, we observed that lowering the temperature from 37 °C to 22 °C did not impair release of stored Ca^{2+} in endothelial cells (Figures 4a and 4b). The time to reach peak $[Ca^{2+}]_i$ was 0.35 min at either temperature. Re-addition of extracellular Ca^{2+} led to Ca^{2+} entry at both temperatures. The time to reach peak $[Ca^{2+}]_i$ after addition of Ca^{2+} -complete buffer was 0.52 min at 37 °C and 0.86 min at 22 °C. The peak $[Ca^{2+}]_i$ value reached at the lower temperature was higher after both mobilization of the intracellular Ca^{2+} pool (489 ± 20 nM at 37 °C, versus 608 ± 22 nM at 22 °C; $P < 0.01$, $n = 8$) and after Ca^{2+} entry (693 ± 68 nM at 37 °C, versus 753 ± 40 nM at 22 °C; $P < 0.05$, $n = 8$). Again as in Figure 1, the return of $[Ca^{2+}]_i$ to resting levels was slower at the lower temperature. These data suggest that both components of Ca^{2+} mobilization (release and influx) are intact at 22 °C and are

Figure 4 Effect of temperature on Ca^{2+} release and Ca^{2+} entry in intact cells

Fura-2-loaded cells were perfused with Ca^{2+} -free buffer containing 1 mM EGTA. BK (1 μ M) was added to mobilize the Ins(1,4,5) P_3 -sensitive intracellular pool. As indicated, Ca^{2+} -complete buffer containing 1.4 mM Ca^{2+} was rapidly added to the cells to initiate Ca^{2+} entry. Buffer temperature was either 37 °C or 22 °C. Data given as means $\pm$ S.E.M. ($n = 8$ for 22 °C; $n = 10$ for 37 °C). Peak $[Ca^{2+}]_i$ levels reached at 22 °C were significantly higher than those reached at 37 °C ($P < 0.01$).

Figure 6 Effect of temperature on TG-induced Ca^{2+} mobilization in endothelial cells

Fura-2-loaded cells were perfused with buffer at either 37 °C or 22 °C and stimulated with 0.2 μM TG as indicated. (a) Cells were perfused with Ca^{2+} -complete buffer. (b) Cells were perfused with Ca^{2+} -free buffer containing 1 mM EGTA. (c) Cells were perfused with Ca^{2+} -complete buffer. Ca^{2+} -insensitive fluorescence of cells excited at 360 nm was monitored continuously. As indicated, buffer was changed to nominally Ca^{2+} -free buffer containing 200 μM MnCl_2 and quenching of the fura-2 signal was monitored. Data are given as means $\pm$ S.E.M. ($n = 3$ for each).

Figure 7 Effect of temperature on BK-induced arachidonic acid release

Cells were labelled with [^3H]arachidonic acid. (a) Labelled cells grown on beads were placed in columns, perfused at 1 ml/min with HBSH containing 0.25% fatty-acid-free BSA and stimulated with 1 μM BK. Data given as means $\pm$ S.E.M. ($n = 3$). (b) Labelled cells grown in flasks were stimulated with 1 μM A23187 and samples were taken to determine arachidonic acid release. Data given as means $\pm$ S.E.M. ($n = 4$).

consistent with previous data which show that BK activates phospholipase C and initiates InsP_3 formation at 22 °C.

The peak $[\text{Ca}^{2+}]_i$ achieved after agonist stimulation and the duration of the response are regulated by the activity of Ca^{2+} -ATPases. These enzymes lower $[\text{Ca}^{2+}]_i$ by sequestering Ca^{2+} in the endoplasmic reticulum and by pumping Ca^{2+} out of the cell. Impairment of Ca^{2+} -ATPase activity may go undetected when measuring $[\text{Ca}^{2+}]_i$ if changes in either Ca^{2+} release or Ca^{2+} entry occur concomitantly. To begin to address this issue, we have measured BK-induced Ca^{2+} entry, using $^{45}\text{Ca}^{2+}$. As shown in Figure 5, Ca^{2+} uptake occurs rapidly at both 37 °C and 22 °C. However, the initial rate of uptake is approx. 2-fold lower at 22 °C than at 37 °C. These data are consistent with those in Figure 3, which show that peak $[\text{Ca}^{2+}]_i$ is reached more slowly at 22 °C and suggests that a decrease in the rate of Ca^{2+} entry may be involved.

In previous studies [8], we have shown that TG, a selective inhibitor of the Ca^{2+} -ATPase on the endoplasmic reticulum [19],

mobilizes the intracellular Ca^{2+} pool in endothelial cells and activated Ca^{2+} entry. A number of cells respond to TG in a similar fashion, and the concept has emerged that the status of the intracellular pool regulates the activity of the Ca^{2+} -entry mechanisms [20–22]. Accordingly, an agonist such as BK discharges the intracellular Ca^{2+} pool and thereby activates Ca^{2+} entry. If this is true, then elevation of $[\text{Ca}^{2+}]_i$ by either TG or BK should occur by similar mechanisms and should be similarly affected by temperature. To examine this, cells were challenged with TG and $[\text{Ca}^{2+}]_i$ was monitored. As shown in Figure 6(a), TG elevated $[\text{Ca}^{2+}]_i$ at both temperatures and in each case $[\text{Ca}^{2+}]_i$ remained elevated for a prolonged period of time. Consistent with our findings above, TG stimulated a larger rise in $[\text{Ca}^{2+}]_i$ at 22 °C than at 37 °C (833 ± 22 nM versus 610 ± 50 nM at 14 min; $P < 0.01$). When these experiments were repeated in the presence of 1 mM EGTA, we found no significant difference in the ability of TG to mobilize the intracellular Ca^{2+} pool at either temperature (Figure 6b). It has been previously reported that the Ca^{2+} -entry

pathway activated by TG or BK in endothelial cells is also permeable to Mn^{2+} [23]. As Mn^{2+} enters the cell, fura-2 fluorescence is quenched. In the following studies, TG was added to cells before Mn^{2+} to mobilize the intracellular Ca^{2+} pool and activate the influx pathway. Subsequently Mn^{2+} was added, and Mn^{2+} entry was measured by monitoring quenching of fura-2 at 360 nm (isosbestic point of Ca^{2+} -sensitive fura-2 spectrum) (Figure 6c). Lowering the temperature decreased Mn^{2+} entry by 1.5-fold during the first 20 s. Since we have previously reported that the prolonged elevation of $[Ca^{2+}]_i$ after TG is primarily due to Ca^{2+} entry from outside the cell [8], it is evident that TG mobilized the intracellular Ca^{2+} pool and activated influx at both temperatures.

Next we looked at the effect of temperature on BK-stimulated arachidonic acid release. Cells grown on Cytodex 3 beads and labelled [3H]arachidonate were placed in a flow-through column to determine label release over time. At 37 °C, BK stimulated a rapid release of label, which reached a peak rate of 1399 c.p.m./min per 10^6 cells by 2 min (Figure 7). The rate gradually slowed, returning to baseline at 16 min. At 22 °C, BK also stimulated label release; however, the maximum rate was only 710 c.p.m./min per 10^6 cells and occurred 3.5 min after addition of the agonist. Thus, although BK was able to activate arachidonic acid release, the activation process was impaired. To show more clearly that Ca^{2+} -activated phospholipase A_2 is impaired at 22 °C, we investigated A23187-stimulated arachidonic acid release from cells in flasks. As seen in Figure 7(b), A23187-stimulated arachidonic acid release both is slower and occurs to a limited extent at 22 °C. The maximum rate of release for cells at 37 °C was 1405 c.p.m./min per 10^6 cells, reached at 3 min after addition of the agonist, and for cells at 22 °C it was 705 c.p.m./min per 10^6 cells reached at 5 min.

DISCUSSION

In this study we have investigated the effects of lower temperature on second-messenger systems involved in arachidonic acid release from vascular endothelial cells. Previous work had shown that lowering the temperature from 37 °C to 30 °C completely prevented BK-induced $Ins(1,4,5)P_3$ formation without blocking arachidonic acid release [14]. This result led to the suggestion that phospholipase A_2 activation in endothelial cells is independent of phospholipase C activation and, since $Ins(1,4,5)P_3$ is not formed, independent of Ca^{2+} mobilization. However, in the present studies we found that $[Ca^{2+}]_i$ was rapidly elevated by BK in cells incubated at either 37 °C or 22 °C. When we corrected $[Ca^{2+}]_i$ for the temperature-related change in K_d for Ca^{2+} binding to fura-2 [17], $[Ca^{2+}]_i$ responses at 22 °C were larger (peak) and returned to basal levels more slowly, suggesting an effect of temperature on mechanisms which return elevated $[Ca^{2+}]_i$ to baseline. Resting $[Ca^{2+}]_i$ was not affected by temperature.

The rise in $[Ca^{2+}]_i$ in response to BK is the summation of both Ca^{2+} release from intracellular stores and Ca^{2+} influx from outside the cell [6–8]. As in many other cell types, mobilization of Ca^{2+} from intracellular stores is initiated in response to $Ins(1,4,5)P_3$ formation. In addition, it has recently been reported that $Ins(1,3,4,5)P_4$ activates Ca^{2+} entry in vascular endothelial cells [24]. These inositol phosphates are formed after activation of phospholipase C. Since we observed a substantial rise in $[Ca^{2+}]_i$ at 22 °C in response to BK, it appears, in contrast with previous studies [14], that phospholipase C is activated at lower temperatures. This was substantiated in experiments using [3H]-inositol-labelled cells, where we found that BK stimulated $Ins(1,4,5)P_3$ formation at 22 °C. The time course for $InsP_3$

accumulation was not different from that at 37 °C; however, total $InsP_3$ formation was decreased and total inositol phosphate production (indicative of phospholipase C activity) was approx. 3-fold lower.

Using a permeabilized cell model, we showed that $Ins(1,4,5)P_3$ effectively induced Ca^{2+} mobilization from intracellular sites at both temperatures. This is in agreement with other studies which also show that $Ins(1,4,5)P_3$ -induced Ca^{2+} release in permeabilized cell systems is relatively insensitive to temperature [15,16]. Typical responses to $Ins(1,4,5)P_3$ in permeabilized cells are transient, due to continuous activity of the Ca^{2+} -ATPase on the endoplasmic reticulum and to the activity of inositol phosphate phosphatases which rapidly degrade added $Ins(1,4,5)P_3$. However, we observed a more sustained Ca^{2+} response to $InsP_3$ at 22 °C than at 37 °C. A portion of the sustained Ca^{2+} rise could be accounted for by impaired Ca^{2+} -ATPase activity and decreased ability to return mobilized Ca^{2+} to the intracellular pool. It also is possible that a temperature-sensitive phosphatase(s) may alter the metabolism of inositol phosphates and allow $Ins(1,4,5)P_3$ levels to remain relatively high at 22 °C by slowing its metabolism.

Since $Ins(1,4,5)P_3$ generation and $Ins(1,4,5)P_3$ activity at its intracellular receptor occur at 22 °C, one would expect that BK mobilizes the intracellular Ca^{2+} pool in intact cells. In experiments in which we dissociated intracellular Ca^{2+} mobilization from Ca^{2+} entry, we found that BK mobilized intracellular Ca^{2+} and elevated $[Ca^{2+}]_i$ at 22 °C. Interestingly, the peak response was significantly higher at 22 °C than at 37 °C. Our data showing impaired [3H] $Ins(1,4,5)P_3$ formation at 22 °C suggest it is unlikely that the larger peak response is due to a faster rate of formation of $Ins(1,4,5)P_3$ or to a larger amount of $Ins(1,4,5)P_3$ formed. Rather, this effect is likely to be due to impairment of mechanisms which decrease $[Ca^{2+}]_i$. In endothelial cells, $[Ca^{2+}]_i$ returns to basal levels through the activity of Ca^{2+} -ATPases on the endoplasmic reticulum and the plasma membrane. Although Na^+/Ca^{2+} exchange may be involved in regulation of $[Ca^{2+}]_i$ in other cells, a role for this mechanism has not been demonstrated in endothelial cells. It appears likely that the higher peak $[Ca^{2+}]_i$ and sustained elevation above basal levels seen with both BK and TG results from temperature effects on Ca^{2+} -ATPase(s). The importance of Ca^{2+} -ATPase inhibition at lowered temperatures may be masked by alterations in Ca^{2+} entry if only changes in $[Ca^{2+}]_i$ are monitored. For example, if Ca^{2+} entry is decreased, a larger peak $[Ca^{2+}]_i$ and delayed return to baseline would suggest inhibition of Ca^{2+} -ATPase activity. In experiments in which we dissociated intracellular Ca^{2+} release from Ca^{2+} entry, it is clear that Ca^{2+} -entry mechanisms are active at 22 °C and lead to a larger, more prolonged, elevation of $[Ca^{2+}]_i$. When we directly investigated the effects of temperature on Ca^{2+} entry, we found that BK-induced $^{45}Ca^{2+}$ entry was approx. 2-fold slower at 22 °C. Since Ca^{2+} entry is significantly decreased at 22 °C, temperature effects on the Ca^{2+} -ATPase activity on the plasma membrane most probably represent an important determinant of agonist-induced $[Ca^{2+}]_i$ at 22 °C. In agreement with these findings, TG produced a larger rise in $[Ca^{2+}]_i$ at 22 °C. Since TG-induced Mn^{2+} entry is inhibited initially, the major mechanism again appears to be inhibition of the Ca^{2+} -ATPase on the plasma membrane (TG inhibits the Ca^{2+} -ATPase on the endoplasmic reticulum). In addition to inhibition of the Ca^{2+} -ATPase on the plasma membrane, we saw a small but consistent decrease at 22 °C in the rate of Ca^{2+} sequestration into intracellular stores, presumably due to decreased activity of the Ca^{2+} -ATPase on the endoplasmic reticulum. Recent studies using avian nasal-gland cells also showed temperature-related changes in $[Ca^{2+}]_i$ [17]. Lowering the temperature from 38 °C to 27 °C did not inhibit agonist-induced elevation of $[Ca^{2+}]_i$ in these studies. Rather, as the temperature

was lowered, decreases in Ca^{2+} entry and Ca^{2+} efflux were seen, leading to a higher peak and sustained elevation of $[\text{Ca}^{2+}]_i$.

Peak $[\text{Ca}^{2+}]_i$ was attained more slowly at 22 °C. This could potentially result from impairment of phospholipase C and $\text{Ins}(1,4,5)\text{P}_3$ formation, from a decrease in the rate of Ca^{2+} entry, or from delayed activation/impaired activity of Ca^{2+} -ATPase(s). Since each is affected by lowering the temperature, the delay in reaching peak $[\text{Ca}^{2+}]_i$ probably represents the sum of these effects of reduced temperature.

Ca^{2+} entry is regulated by the status of the intracellular Ca^{2+} pool, such that emptying the pool leads to Ca^{2+} influx [8,20–22]. TG mobilizes Ca^{2+} pools and activates Ca^{2+} entry in endothelial cells [8]. Whether the influx pathway utilized by an agonist is identical with that activated in response to TG is uncertain. In this paper we wished to see whether temperature might differentially alter Ca^{2+} entry in response to BK or TG. A qualitatively similar pattern was seen with both agonists. We saw higher levels of $[\text{Ca}^{2+}]_i$ in response to both agonists at 22 °C, and we saw inhibition of both BK-induced $^{45}\text{Ca}^{2+}$ entry and TG-induced Mn^{2+} entry. Although this does not prove that the entry mechanisms are identical, it does show that both are similarly temperature-sensitive.

It is clear that BK effectively elevates $[\text{Ca}^{2+}]_i$ in endothelial cells at lower temperatures. One would expect that those mechanisms which are dependent on $[\text{Ca}^{2+}]_i$ would also be stimulated, even though low temperature may decrease their activity. When we monitored arachidonic acid release from endothelial cells, we found that the initial rate and extent of release was inhibited at 22 °C. In other experiments in which A23187 was used to elevate Ca^{2+} directly, similar results were obtained. This inhibition of phospholipase A_2 activity occurred under conditions where $[\text{Ca}^{2+}]_i$ was above normal levels, suggesting that temperature directly alters phospholipase A_2 activity. It is not possible to determine from our data whether the effect of temperature is on the interaction of the lipid substrate with the enzyme or is due to some direct change in the enzyme protein. These data are consistent with those previously published which show that BK stimulates arachidonic acid release at lower temperatures [14]; however, the conclusions are different, since we also documented that both $\text{Ins}(1,4,5)\text{P}_3$ and $[\text{Ca}^{2+}]_i$ are elevated. We have recently reported that Ca^{2+} -dependent and Ca^{2+} -independent mechanisms for phospholipase A_2 activation exist in endothelial cells and that BK exclusively utilizes Ca^{2+} -dependent pathways [9]. Thus one would expect activation of phospholipase C and generation of inositol phosphates to be a required step in BK-stimulated arachidonic acid release. In fact, our present data clearly show that phospholipase C is activated at lower temperatures and that all the signals necessary for Ca^{2+} -activated arachidonate release are present at lower temperatures. This is in contrast with a

previous report, which suggests that BK-stimulated arachidonic acid release was independent on phospholipase C [14].

Although the signalling pathways from BK to arachidonic acid release appear to be intact in endothelial cells exposed to lower temperature, there are numerous important effects of temperature, and each should be considered in interpreting data from studies done at room temperature. These effects include partial inhibition of phospholipase C, Ca^{2+} -ATPase(s), Ca^{2+} -entry mechanisms, phospholipase A_2 and perhaps inositol phosphatases. The outcome of the complex interactions among these effects is a higher and more prolonged elevation of $[\text{Ca}^{2+}]_i$ and a decrease in arachidonic acid release in response to an agonist.

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