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Bradykinin-evoked Ca^{2+} mobilization in Madin Darby canine kidney cells

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Abstract

We studied the mechanisms underlying the bradykinin-evoked changes in intracellular calcium concentration ($[Ca^{2+}]_i$) in Madin Darby canine kidney (MDCK) cells. Bradykinin evoked a $[Ca^{2+}]_i$ transient in a dose-dependent manner, measured by fura-2 fluorimetry and digital video imaging. The transient consisted of a rise and a decay and $[Ca^{2+}]_i$ returned to baseline without oscillations. External Ca^{2+} influx occurred, as demonstrated by Mn^{2+} quench and external Ca^{2+} removal measurements. Bradykinin acted by stimulating bradykinin B_2 receptors as evidenced by blockade by D-arginyl-L-arginlyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α,3β,7aβ)-octahydro-1*H*-indole-2-carbonyl-L-arginine (HOE 140) but not by D-arginyl-L-arginlyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α,3β,7aβ)-octahydro-1*H*-indole-2-carbonyl ([Des–Arg]HOE 140). The $[Ca^{2+}]_i$ signal was abolished by 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122) and partially inhibited by neomycin, implying mediation by phospholipase C. The transient was initiated by a release of Ca^{2+} from internal stores since it was abolished by pretreatment with thapsigargin or cyclopiazonic acid. The mobilization of the internal Ca^{2+} store subsequently triggered a 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SKF 96365)-insensitive Ca^{2+} entry. Pretreatment with carbonylcyanide *m*-chlorophynylhydrozone and gly-phe-β-naphthylamide did not alter the transient, thus excluding the participation of mitochondria and lysosomes. Efflux via Ca^{2+} pumps contributed to the decay of the transient. Efflux via Na^+/Ca^{2+} exchange or sequestration by mitochondria and lysosomes was insignificant. The transient was blunted by the protein kinase C activator phorbol 12-myristate 13-acetate, and was enhanced by the protein kinase C inhibitors sphingosine and chelerythrine, the protein kinase A inhibitor 2,5-di-(*t*-butyl)-1,4-hydroquinone, *N*-[2-(*p*-bromocinnamylamino)ethyl]5-isoquinolinesulfonamide (H-89), the agent 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8), and agents that elevated levels of 3′,5′-cyclic guanosine monophosphate. The transient did not heterologously desensitize with that evoked by ATP, ADP or UTP.

Keywords: Bradykinin; MDCK cells; Ca^{2+}, thapsigargin; Ca^{2+} stores; Fura-2

1. Introduction

In many excitable and non-excitable cells, bradykinin has been shown to interact with plasmalemmal receptors (mainly bradykinin B_2 subtype) and to elevate inositol 1,4,5-trisphosphate (IP_3) levels by activating the G-protein–phospholipase C system, resulting in Ca^{2+} release from internal stores and an increase in $[Ca^{2+}]_i$ (Quitterer et al., 1995; Berridge, 1993). In some non-excitable systems such as endothelial cells (Mendelowitz et al., 1992), the bradykinin-induced depletion of internal stores activates capacitative Ca^{2+} entry (Putney and Bird, 1993), whereas in other systems, such as neuroblastoma cells, bradykinin does not (Coggan and Thompson, 1995).

In Madin Darby canine kidney (MDCK) epithelial cells, bradykinin has been shown to activate bradykinin B_2 receptors (Coyne et al., 1989), stimulate IP_3 accumulation (Portilla and Morrison, 1986; Aboolian et al., 1989; Lang et al., 1991) and elevate $[Ca^{2+}]_i$ (Pidikiti et al., 1985; Aboolian et al., 1989; Coyne et al., 1989; Lang et al., 1991; Lou et al., 1992; Vamos et al., 1995), which in turn opens Ca^{2+}-activated K^+ channels and results in cell hyperpolarization (Lang et al., 1991). Bradykinin also stimulates an inward short-circuit current and causes chloride secretion (Simmons, 1992), a decrease in membrane

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resistance (Ritter and Lang, 1991), and an increased synthesis of cAMP (Hassid, 1983) and prostaglandins (Coyne et al., 1989).

It has been reported that bradykinin triggers a biphasic $[Ca^{2+}]_i$ transient which consists of a peak and decay (Pidikiti et al., 1985; Aboolian et al., 1989; Lang et al., 1991; Lou et al., 1992; Vamos et al., 1995), that maintenance of the bradykinin response depends on extracelluar Ca^{2+} (Vamos et al., 1995), and that the $[Ca^{2+}]_i$ increase shows protein kinase C-dependent homologous desensitization (Lou et al., 1992). However, many important issues remain to be clarified: 1) The Ca^{2+} source (s): Is there Ca^{2+} influx? What is the contribution of Ca^{2+} influx compared with Ca^{2+} release from internal stores? What Ca^{2+} stores, e.g., the endoplasmic reticulum, mitochondria, nuclei and lysosomes, are involved? 2) How does the $[Ca^{2+}]_i$ transient decay? Extrusion via Ca^{2+} pump and/or Na^+/Ca^{2+} exchange? Or sequestration by organelles? 3) How is the $[Ca^{2+}]_i$ transient affected by agents that alter levels of second messengers such as cGMP, cAMP, protein kinase C and protein kinase A? In this study we provide evidence which might answer these questions.

2. Methods

2.1. Cell culture

MDCK cells obtained from American Type Culture Collection (CRL-6253, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2-containing humidified air.

2.2. Solutions

Normal buffer (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 2; Hepes 10; glucose 5. Ca^{2+}-free buffer contained no Ca^{2+} plus 1 mM EGTA (calculated $[Ca^{2+}] < 0.1$ nM). In Na^+-free buffer, Na^+ was isomolarly replaced with *N*-methyl-glucamine. The experimental solution contained $\leq 0.1\%$ of solvent (dimethyl sulfoxide or ethanol) which did not affect $[Ca^{2+}]_i$ ($n = 6$).

2.3. Optical measurements of $[Ca^{2+}]_i$

2.3.1. Fluorimetry

Trypsinized cells (10^6/ml) were loaded with 2 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*,*N*-tetraacetic acid pentaacetoxymethyl ester (fura-2/AM) for 30 min at 25°C in DMEM. Cells were washed and resuspended in normal buffer and were washed again before each experiment to minimize extracellular dye. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette normally contained 1 ml of buffer and 0.5 million of cells unless otherwise stated. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer (Japan) by continuously recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-s intervals. Maximal and minimal fluorescences were obtained by adding TX-100 (0.1%) and EGTA (20 mM) sequentially at the end of experiment. The ratio of excitation signals at 340 and 380 nm was used to calculate $[Ca^{2+}]_i$ as described previously (Grynkiewicz et al., 1985). Mn^{2+} quench experiments were performed in normal buffer containing 50 μM of $MnCl_2$ by recording the excitation signals at 340, 360, and 380 nm and emission signal at 510 nm, which were continuously alternated at 1-s intervals. Preliminary experiments showed that trypsinized cells yielded qualitatively similar results as cells attached to coverslips. We decided to use trypsinized cells because this procedure is easier and less time-consuming.

2.3.2. Digital video imaging

Suspended cells were seeded on polylysine-coated coverslips (22 mm, No. 1) at a density allowing imaging of ~30 single cells. The attached cells were loaded with 2 μM fura-2/AM for 30 min at 25°C in normal buffer. The coverslip was transferred to a chamber (25°C) on the stage of a Nikon Diaphot microscope and viewed under bright light and UV illumination via a 40× (1.3 NA) oil immersion fluorescence objective (Nikon Fluor). Solutions were applied as a 3-ml bolus to the chamber (containing 0.5 ml of solution). This allowed rapid and complete change of the solution (removed by aspiration as the volume of the chamber solution exceeded 0.5 ml for the new solution).

The MiraCal imaging system (Life Science Resources, Cambridge, UK) was used in conjunction with a slow scan cooled CCD camera (CMCA Nikon DF/SB 0.45× WILDFIELD) for digital video imaging of changes in $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was calculated to 8-bit accuracy (256 grey levels) every 1.5 s. Data were analyzed off-line for real changes in $[Ca^{2+}]_i$ by examination of the 340 and 380 nm of excitation signals. The emission wavelength was detected at 510 nm. Ratio values were converted to an estimate of $[Ca^{2+}]_i$ by using the formula: $[Ca^{2+}]_i = K_d \beta (R - R_{min})/(R_{max} - R)$.

Calibration constants were obtained by addition of 10 μM ionomycin in solutions containing 2 mM Ca^{2+} ($R_{max} = 2.1$), and no added Ca^{2+} plus 20 mM EGTA ($R_{min} = 0.2$). The value of β (equivalent to 380 nm at R_{min}/380 nm at R_{max}) is 3.13 and a K_d (dissociation constant) of 155 nM was assumed (Mason et al., 1993).

All experiments were performed at room temperature (23–25°C).

2.4. Chemical reagents

The reagents for cell culture were from Gibco (Grand Island, NY, USA). Fura-2/AM was from Molecular Probes

(Eugene, OR, USA). Bradykinin, D-arginyl-L-arginlyl-L-prolyl-*trans*-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbo-nyl-L-(2α,3β,7aβ)-octahydro-1*H*-indole-2-carbonyl-L-argi-nine (HOE 140), D-arginyl-L-arginlyl-L-prolyl-*trans*-4-hydroxy-L-prolyl-glycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α,3β,7aβ)-octahydro-1*H*-indole-2-carbonyl ([Des–Arg]HOE 140), phorbol 12-myristate 13-acetate, sphingosine, chelerythrine, 8-(diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8), diethylamine/nitric oxide complex sodium, diethylenetriamine/nitric oxide adduct were from RBI (Natick, MA, USA). 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyr-role-2,5-dione (U73122), 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione (U73343), 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SKF 96365), 2,5-di-(*t*-butyl)-1,4-hydroquinone, *N*-[2-(*p*-bromocinnamylamino)ethyl]5-isoquinolinesulfonamide (H-89), 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP), 8-bromoguanosine-3′-5′-cyclic monophosphate (8-Br-cGMP), and (*S*)-5-isoquinolinesulfonic acid, 4-[2-[(5-isoquinolinyl-sulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-pi-perazinyl)-propyl]phenyl ester (KN-62) were from Biomol (Pittsburgh, PA, USA). Other reagents were from Sigma (St. Louis, MO, USA).

2.5. Statistical analysis

For fluorimetry experiments, the recordings are typical of 5–6 experiments. For imaging experiments, the recordings are typical of ~40 cells. All values are reported as means ± S.E. Statistical comparisons were determined by using the Student's *t*-test, and significance was accepted when $P < 0.05$.

3. Results

3.1. Bradykinin evokes a non-oscillatory $[Ca^{2+}]_i$ transient

$[Ca^{2+}]_i$ changes were measured in suspended or single cells in the presence of 2 mM extracellular Ca^{2+}, using the Ca^{2+}-sensitive dye fura-2. Resting $[Ca^{2+}]_i$ was 50–60 nM. Fig. 1B and C illustrate representative responses evoked by bradykinin. Bradykinin evoked a rapid increase in $[Ca^{2+}]_i$ followed by a gradual decay and $[Ca^{2+}]_i$ returned to baseline in ~3 min despite the continued presence of the hormone. Fig. 1A shows that this response was dose dependent between 10^{-9}–10^{-4} M, with a maximum peak occurring at 10^{-8} M (610 ± 23 nM, $n = 6$); the bradykinin responses at higher doses were blunted. Bradykinin at 10^{-10} M had little effect on $[Ca^{2+}]_i$. Digital video imaging of $[Ca^{2+}]_i$ in single cells revealed that bradykinin elevated $[Ca^{2+}]_i$ in >99% of cells examined ($n = \sim 600$ cells). The $[Ca^{2+}]_i$ response was similar to that recorded from cell suspensions, and no oscillations were ever observed (see Fig. 3E for examples). Further, for responses induced by higher doses of bradykinin (10^{-7}–10^{-4} M), the time needed to reach the peak was 10 ± 2 s ($n = 5$) whereas for responses induced by lower doses, a much longer time was needed (18 ± 2 s for 10^{-8} M and 47 ± 2 s for 10^{-9} M, $n = 5$, not shown). However, the time to peak and the duration of the decay of the $[Ca^{2+}]_i$ transient are difficult to assess when $[Ca^{2+}]_i$ is measured in a population of cells because we found, during Ca^{2+} imaging measurements, that not all cells responded at the same time to an agonist. Nevertheless, our results reflected the average response of a population of cells. In the following experiments with bradykinin, the submaximal concentration of 1 μM was used unless otherwise stated.

3.2. Internal release and external influx both contribute to the bradykinin response

To examine the contribution of extracellular Ca^{2+} to the bradykinin response, we stimulated suspended cells in the absence of extracellular Ca^{2+}. For Ca^{2+} removal, some laboratories centrifuge and suspend cells in Ca^{2+}-free plus EGTA buffer before the experiments. We avoided this procedure because cells bathed in Ca^{2+}-free buffer for a long time might be depleted of internal Ca^{2+}. Instead, 0.1 ml of cell suspension was added to 0.9 ml of Ca^{2+}-free buffer (no added Ca^{2+} plus 1 mM EGTA) 1 min before bradykinin stimulation. This yielded a calculated extracellular $[Ca^{2+}] < 0.1$ nM. Fig. 1B depicts that the peak amplitude of the bradykinin-induced $[Ca^{2+}]_i$ increase was reduced by Ca^{2+} removal by ~48% ($n = 6$; $P < 0.05$), implying that Ca^{2+} influx occurred early during bradykinin stimulation. The fact that Ca^{2+} removal did not alter resting $[Ca^{2+}]_i$ indicated that the amount of fura-2 leakage in our experiments was negligible. After the bradykinin response had decayed, thapsigargin, which inhibits the endoplasmic reticulum Ca^{2+} pump, thus allowing Ca^{2+} to leak from the endoplasmic reticulum stores (Thastrup et al., 1990), was added and induced a $[Ca^{2+}]_i$ transient which exhibited slower rise and decay kinetics than the transient elicited by bradykinin. Likewise, the thapsigargin-induced $[Ca^{2+}]_i$ increases were inhibited by ~50% by Ca^{2+} removal. With the bradykinin response, while Ca^{2+} removal resulted in a reduced peak and a slower decay, the time needed to return to baseline was not altered. However, with the thapsigargin response, Ca^{2+} removal abolished the plateau that was sustained in the presence of Ca^{2+}. This suggests that in the presence of Ca^{2+}, bradykinin induced Ca^{2+} influx early during stimulation which lasted about 3 min. In contrast, thapsigargin induced a sustained Ca^{2+} influx (>8 min).

We used another maneuver to prove that Ca^{2+} influx really occurred by using Mn^{2+} as a surrogate for Ca^{2+}. Mn^{2+} enters cells through similar pathways as Ca^{2+}, but

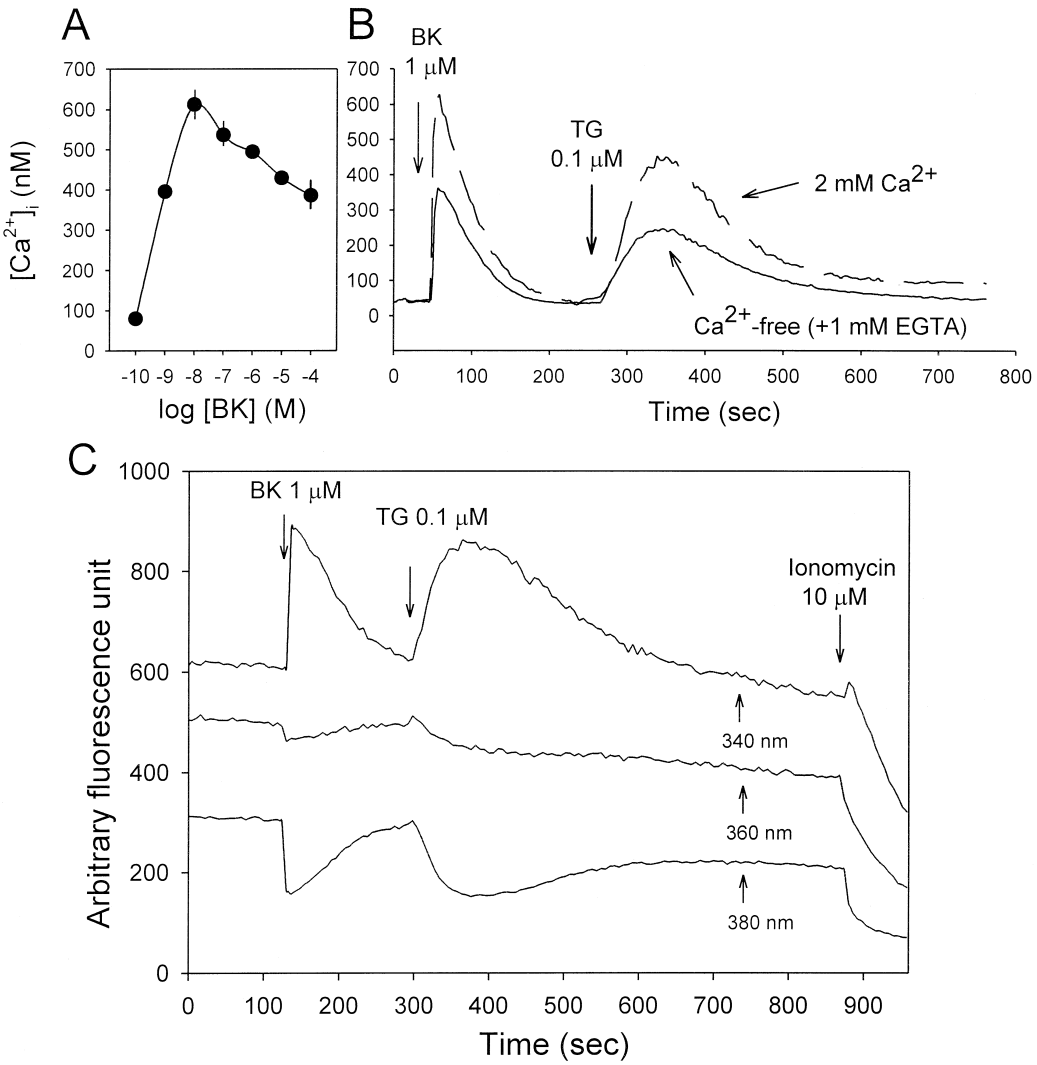

Fig. 1. A, Dose-response curve of the peak amplitude of the bradykinin (BK)-induced increases in $[Ca^{2+}]_i$ in a population of MDCK cells suspended in normal buffer. Data are means ± S.E. Some error bars are invisible because they are smaller than the symbols. B, Bradykinin- and thapsigargin (TG)-induced increases in $[Ca^{2+}]_i$ in normal buffer (dashed line) or Ca^{2+}-free buffer (solid line) which contained no added Ca^{2+} plus 1 mM EGTA. C, Bradykinin- and thapsigargin-induced Ca^{2+} influx detected by Mn^{2+} quench measurements. $MnCl_2$ (50 μM) was added to cells 90 s prior to bradykinin stimulation. Excitation signals, which alternated at 340, 360 and 380 nm, and emission signal at 510 nm were continuously recorded at 1-s intervals. All traces are typical of 5–6 experiments.

quenches fura-2 fluorescence at all excitation wavelengths (Merrit et al., 1989). Thus, Mn^{2+} can aid detection of Ca^{2+} influx. Fluorescence intensity was monitored at the Ca^{2+}-insensitive excitation wavelength of 360 nm and the Ca^{2+}-sensitive wavelengths of 340 nm and 380 nm alternately in normal buffer containing $MnCl_2$ (50 μM). Fig. 1C shows that bradykinin increased the 340 nm signal accompanied by a corresponding drop in the 380 nm signal, and both signals returned to baseline in ~3 min. Concomitantly there was a small but rapid decrease in the 360 nm signal which occurred early after bradykinin addition and gradually returned to baseline as the changes in the 340 nm and 380 nm signals ceased. This provides direct evidence for Ca^{2+} influx. To exclude the possibility that this decrease in the 360 nm signal was an artifact, thapsigargin, which has been shown to trigger a robust increase in $[Ca^{2+}]_i$ in MDCK cells (Jan et al., 1998a,b), was added after the bradykinin response. Thapsigargin induced a gradual increase and decrease of the 340 nm signal, accompanied by a corresponding decrease and increase of the 380 nm signal. Importantly, there was a gradual and lasting decrease in the 360 nm signal, which suggests there was Ca^{2+} influx. The Ca^{2+} ionophore, ionomycin, was added afterwards to ascertain that $MnCl_2$ at 50 μM was able to quench fura-2 fluorescence. Ionomycin elicited a sharp decrease in the 340, 360, and 380 nm signals, showing that the dose of Mn^{2+} was adequate. Thus, it is clear that bradykinin activated Ca^{2+} influx. We tested several Ca^{2+} channel blockers to see if they inhibited Mn^{2+} influx. Pretreatment with the following drugs

for 3–5 min had little effect: nifedipine (10 μM), verapamil (10 μM), diltiazem (10 μM), flunarizine (10 μM), amiloride (10 μM), Ni^{2+} (1 mM), Co^{2+} (1 mM), Cd^{2+} (25 μM), and SKF 96365 (1–30 μM) (not shown; $n = 5$).

3.3. Bradykinin acts by activating bradykinin B_2 receptors

Fig. 2B shows that the bradykinin B_2 receptor inhibitor HOE 140 blocked the 0.1 μM bradykinin-evoked $[Ca^{2+}]_i$ increase. The bradykinin B_1 receptor inhibitor [Des-Arg]HOE 140 in similar experiments caused little inhibition (Fig. 2C), suggesting that bradykinin elevated $[Ca^{2+}]_i$ by activating bradykinin B_2 receptors. Fig. 2B shows that HOE 140 inhibited the bradykinin response competitively since it only partially antagonized the effect of bradykinin at a higher dose (10 μM); additionally, HOE 140 did not affect the $[Ca^{2+}]_i$ increase induced by ATP or thapsigargin. Fig. 2A shows that 10 μM bradykinin added after 0.1 μM bradykinin did not elevate $[Ca^{2+}]_i$, presumably due to homologous desensitization, but there was no heterologous desensitization of the ATP response. Conversely, bradykinin (10 μM) was still able to induce an increase in $[Ca^{2+}]_i$ after pretreatment with HOE 140 and 0.1 μM bradykinin (Fig. 2B). This may be because HOE 140 interfered with the binding of bradykinin at a lower dose and thus prevented bradykinin receptors from desensitizing. Because prostaglandins are released by bradykinin-stimulated MDCK cells (Coyne et al., 1989) and could increase $[Ca^{2+}]_i$ similar to that produced by bradykinin (Aboolian et al., 1989), we tested whether the bradykinin response was mediated by prostaglandins. Incubation of cells for 15 min with indomethacin (10 μM) to inhibit prostaglandin synthesis had no effect on the bradykinin response (not shown; $n = 5$).

3.4. The internal Ca^{2+} source for the bradykinin response is the thapsigargin-sensitive store, but not mitochondria or lysosomes

We demonstrated in Fig. 1B that bradykinin induced internal Ca^{2+} release. The question arose as to which Ca^{2+} stores were involved. We first examined the thapsi-

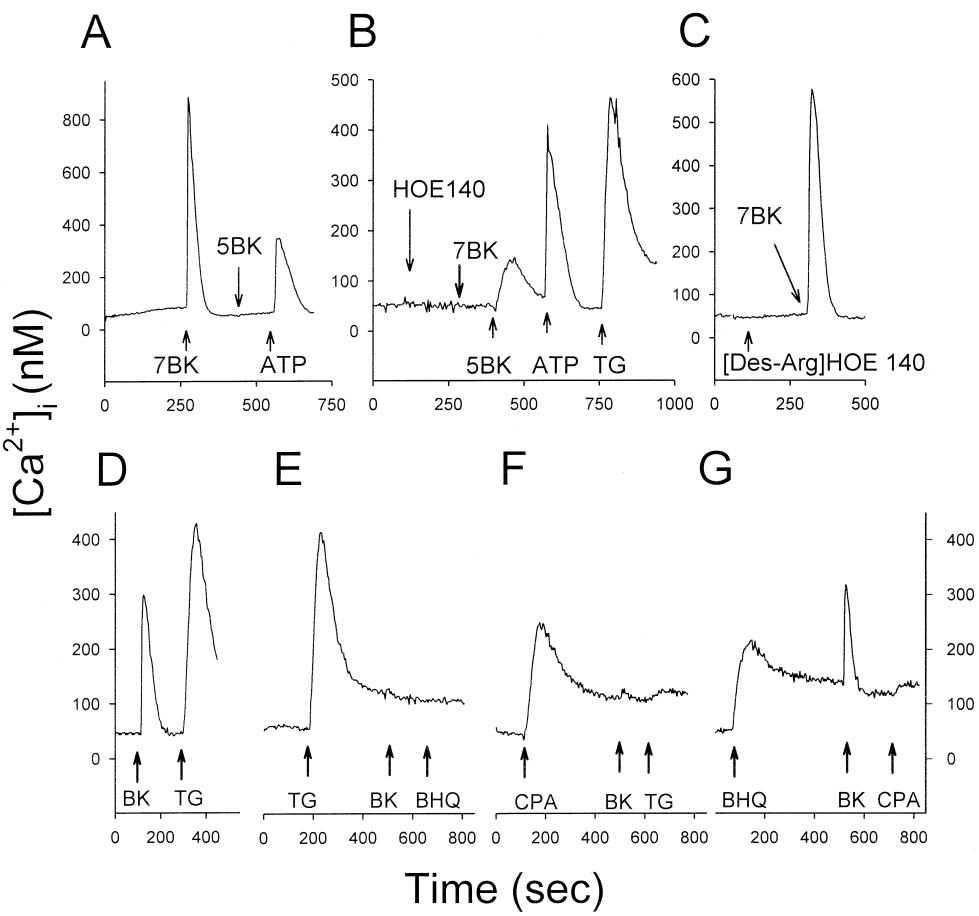

Fig. 2. Bradykinin elevated $[Ca^{2+}]_i$ by activating bradykinin B_2 receptors and releasing Ca^{2+} from the endoplasmic reticulum Ca^{2+} store in a population of MDCK cells suspended in normal buffer. A, Effect of bradykinin (BK; 0.1 μM and 10 μM) and ATP (1 μM) on $[Ca^{2+}]_i$. B, Effect of sequential addition of HOE 140 (0.1 mM), bradykinin (0.1 μM and 10 μM), ATP (1 μM) and thapsigargin (TG; 0.1 μM) on $[Ca^{2+}]_i$. C, Cells were incubated with [Des–Arg]HOE 140 (1 mM) for ~200 s prior to bradykinin (0.1 μM) addition. D–G, Effect of bradykinin (1 μM), thapsigargin (0.1 μM), 2,5-di-(*t*-butyl)-1,4-hydroquinone (BHQ; 50 μM), and cyclopiazonic acid (CPA; 100 μM) on $[Ca^{2+}]_i$. All traces are typical of 5–6 experiments

gargin-sensitive stores. We applied three drugs known to release Ca^{2+} from the endoplasmic reticulum pools by inhibiting the Ca^{2+} pump: thapsigargin, cyclopiazonic acid and 2,5-di-(*t*-butyl)-1,4-hydroquinone (Demaurex et al., 1992). Fig. 2E and F show that both thapsigargin and cyclopiazonic acid induced a $[Ca^{2+}]_i$ transient with a peak amplitude comparable to that evoked by bradykinin with a slower rise, particularly in the case of cyclopiazonic acid, and the plateau was ′100 nM higher than baseline, in contrast to the bradykinin response which lacked a plateau. Bradykinin added after thapsigargin (Fig. 2E) or cyclopiazonic acid (Fig. 2F) failed to elevate $[Ca^{2+}]_i$. 2,5-di-(*t*-butyl)-1,4-hydroquinone added after thapsigargin (Fig. 2E) or thapsigargin added after cyclopiazonic acid (Fig. 2F) did not release more Ca^{2+}. 2,5-Di-(*t*-butyl)-1,4-hydroquinone (50 μM) induced a $[Ca^{2+}]_i$ transient (Fig. 2G) which resembled that induced by cyclopiazonic acid (100 μM); however subsequently added bradykinin still released some Ca^{2+} but cyclopiazonic acid failed to do so. These results suggest that bradykinin, thapsigargin, cyclopiazonic acid and 2,5-di-(*t*-butyl)-1,4-hydroquinone all release Ca^{2+} from the same internal stores. These results combined with that shown in Fig. 1B and C demonstrate that the bradykinin response consists of two sequential steps: Ca^{2+} release from the thapsigargin-sensitive Ca^{2+} stores followed by capacitative Ca^{2+} entry. We next tested whether ryanodine-sensitive Ca^{2+} stores played a role in the bradykinin response. Incubation of cells for 20 min with ryanodine (50 μM) or caffeine (10 mM) did not have an effect ($n = 5$, not shown).

3.5. Mitochondria and lysosomes do not contribute to the increase in the bradykinin response

To further prove that bradykinin elevated $[Ca^{2+}]_i$ by first releasing Ca^{2+} from the thapsigargin-sensitive stores, we examined the contribution of two other Ca^{2+} stores, namely mitochondria and lysosomes. Mitochondria are thought to play an important role in Ca^{2+} homeostasis in chromaffin cells (Herriton et al., 1996), and lysosomes have been reported in MDCK cells to contain Ca^{2+} which is releasable by gly-phe-β-naphthylamide (Haller et al., 1996). Fig. 3B shows that the mitochondrial uncoupler carbonylcyanide *m*-chlorophynylhydrozone caused a small gradual increase in $[Ca^{2+}]_i$ (peak = 150 ± 15 nM; $n = 6$) which slowly decayed to baseline in 300 s. Subsequently added bradykinin and thapsigargin induced $[Ca^{2+}]_i$ increases which were indistinguishable from control. Experiments were carried out with another inhibitor of mitochondria, oligomycin (5 μg/ml). Incubation for 10 min with oligomycin did not alter resting $[Ca^{2+}]_i$ and nor did it affect the responses evoked by bradykinin or thapsigargin

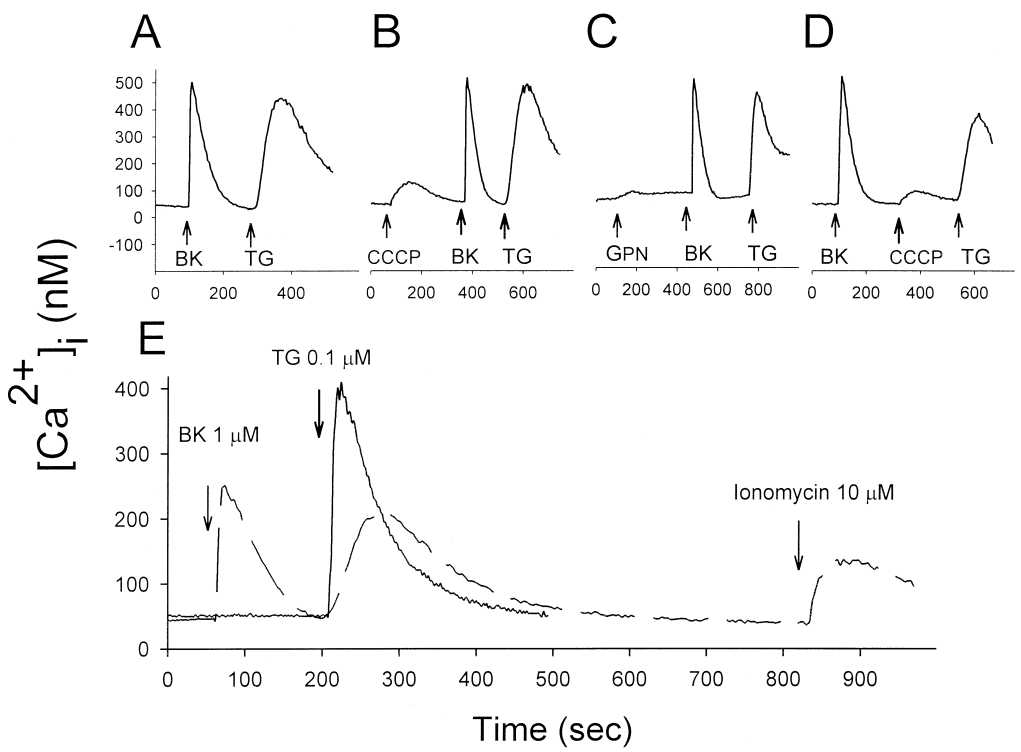

Fig. 3. A–D, Effect of carbonylcyanide *m*-chlorophynylhydrozone (CCCP) and gly-phe-β-naphthylamide (GPN) on $[Ca^{2+}]_i$ in single MDCK cells measured by digital video imaging. The cells were bathed in normal buffer. Concentration: bradykinin (BK) = 1 μM; thapsigargin (TG) = 0.1 μM; CCCP = 2 μM; GPN = 300 μM. E, Cells were bathed in Ca^{2+}-free buffer for 200 s before addition of thapsigargin (solid line) measured by digital video imaging. In a similar experiment (dashed line), cells were bathed in Ca^{2+}-free buffer for 60 s, and then bradykinin, thapsigargin and ionomycin were added. Recordings are representative of 25 cells.

($n = 5$; not shown). Fig. 3C shows that gly-phe-β-naphthylamide induced a small increase in $[Ca^{2+}]_i$ for 400 s without altering the responses induced by bradykinin or thapsigargin. This suggests that the thapsigargin-sensitive Ca^{2+} stores, but not the Ca^{2+} stores in mitochondria or lysosomes, were the internal source for the bradykinin response.

3.6. Decay of the bradykinin response is not due to sequestration by mitochondria or lysosomes

So far, we investigated the mechanisms underlying the rise of the bradykinin response, the next question is: which mechanisms underlie the decay of the transients? In most cells the decay of $[Ca^{2+}]_i$ transients involves buffering by Ca^{2+} binding proteins, efflux via plasmalemmal Ca^{2+} pumps and Na^+/Ca^{2+} exchange, and sequestration by internal stores (Clapman, 1995). Buffering by the nucleus might also play a significant role (Al-Mohanna et al., 1994; Bkaily et al., 1996, 1997a,b). We first determined the contribution of mitochondria and lysosomes. Fig. 3D shows that after the bradykinin response had decayed, carbonylcyanide *m*-chlorophynylhydrozone induced a $[Ca^{2+}]_i$ transient with a peak amplitude no higher than that seen in Fig. 3B. Likewise, oligomycin (5 μg/ml) or gly-phe-β-naphthylamide (300 μM) added after the bradykinin response had the same effect as when they were added before bradykinin (not shown). If the decay of the bradykinin response was due to sequestration by mitochondria and/or lysosomes, then carbonylcyanide *m*-chlorophynylhydrozone, oligomycin and gly-phe-β-naphthylamide should elevate $[Ca^{2+}]_i$ more when they were added after the bradykinin response than when they were added before. Thus, these results suggest that the decay of the bradykinin response was unlikely to involve sequestration by mitochondria and lysosomes.

We next examined whether the decay of the bradykinin response was due to sequestration of Ca^{2+} by thapsigargin-sensitive Ca^{2+} stores. Fig. 3E shows the results of two representative cells from imaging experiments. To avoid refilling of the thapsigargin-sensitive Ca^{2+} stores, these experiments were performed in the absence of extracellular Ca^{2+}. Application of thapsigargin (solid line) induced a sharp increase in $[Ca^{2+}]_i$ which peaked at ~400 nM and subsequently decreased to baseline in ~5 min. As shown in Fig. 1B, the plateau of the thapsigargin response seen in the presence of Ca^{2+} was eliminated by Ca^{2+} removal. This is consistent with the Mn^{2+} quench data (Fig. 1C) and demonstrates that the plateau was due to capacitative Ca^{2+} entry. When the cells were sequentially stimulated with bradykinin and thapsigargin (Fig. 3E; dashed line), the peak amplitude of the thapsigargin-induced $[Ca^{2+}]_i$ transients was reduced by 50%, and the increase was slower. This suggests that the decay of the bradykinin response cannot be solely explained by reuptake of Ca^{2+} by the thapsigargin-sensitive stores. The data, however, do

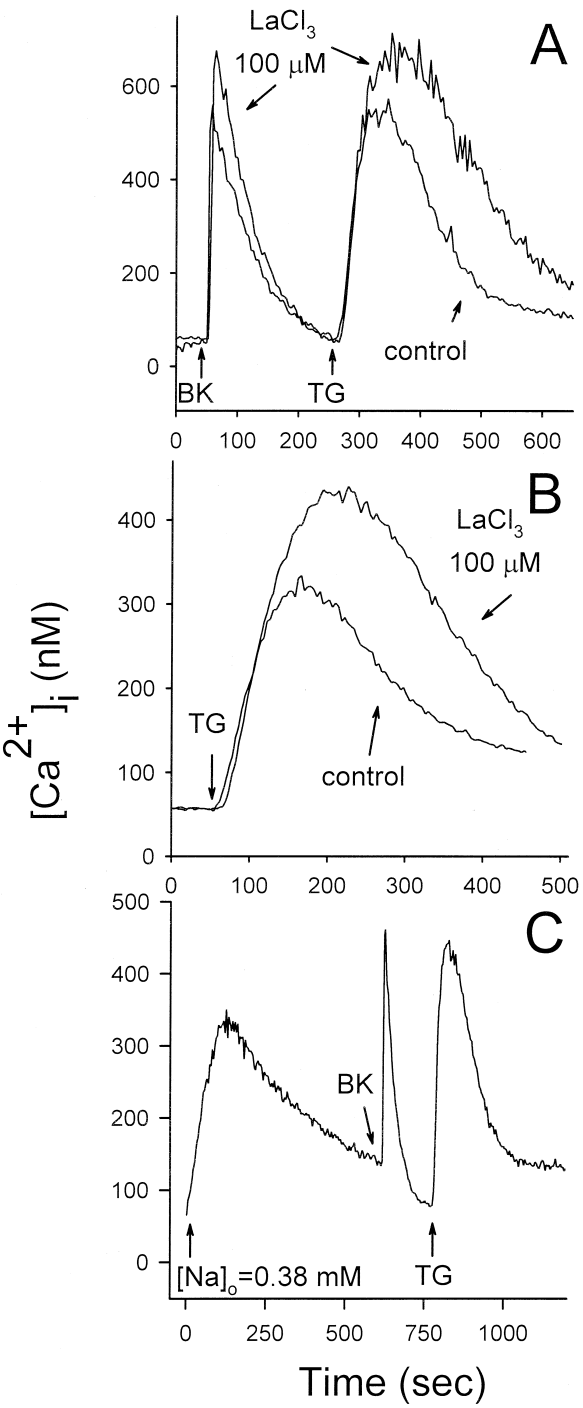

Fig. 4. Mechanisms for the decay of bradykinin-induced increase in $[Ca^{2+}]_i$ in a population of MDCK cells. All the experiments were done in normal buffer. A, Control: bradykinin (BK; 1 μM) and thapsigargin (TG; 0.1 μM) were added at the indicated time. $LaCl_3$ 100 μM: $LaCl_3$ was added to cells 200 s before bradykinin. B, Control: cells were stimulated with thapsigargin (0.1 μM) at the indicated time. $LaCl_3$ 100 μM: $LaCl_3$ was added to cells 200 s before thapsigargin. C, Effect of bradykinin (1 μM) and thapsigargin (0.1 μM) on $[Ca^{2+}]_i$ in reduced extracellular $[Na^+]$. Na^+ was replaced by *N*-methyl-glucamine isomolarly. All traces are typical of 5–6 experiments.

not exclude the possibility that nearly half of the mobilized Ca^{2+} returned to thapsigargin-sensitive stores. We also determined whether there were thapsigargin-insensitive Ca^{2+} stores. Ionomycin added 6 min after the thapsigargin response induced a significant increase in $[Ca^{2+}]_i$ (peak = 130 ± 10 nM; $n = 25$ cells). Thus, the results support the data shown in Fig. 3 (B–D) that there are thapsigargin-inaccessible Ca^{2+} stores, e.g., in mitochondria, lysosomes or even nuclei, in MDCK cells.

3.7. Ca^{2+} efflux via Ca^{2+} pumps, but not via Na^+/Ca^{2+} exchange, plays a significant role in the decay of the bradykinin response

A selective inhibitor is not available for the plasmalemmal Ca^{2+} pump. We tried three manipulations which have been reported to depress the Ca^{2+} pump: external alkalization to pH 8 (Milanick, 1990), addition of eosin (Gatto and Milanick, 1993) or La^{3+} (Milanick, 1990). Eosin (1–10 μM) interfered with fura-2 fluorescence and could not be tested. Fig. 4A shows that La^{3+} (100 μM) pretreatment slightly increased the peak amplitude and significantly increased the area under the curve of the bradykinin response by $36 \pm 2\%$ ($n = 5$; $P < 0.01$). The area under the curve of the transient induced by thapsigargin added after bradykinin (Fig. 4A) or by thapsigargin alone (Fig. 4B) was increased by $99 \pm 5\%$ ($n = 5$; $P < 0.01$). In a separate experiment in which the external medium was alkalized to pH 8 with NaOH, the area under the curve of the bradykinin response was $32 \pm 3\%$ greater than that measured at pH 7.4 (not shown; $n = 6$; $P < 0.05$). Thus, Ca^{2+} efflux via the plasmalemmal Ca^{2+} pump might play a key role in regulating the decay of the bradykinin response.

For Na^+/Ca^{2+} exchange, we lowered external $[Na^+]$ by adding 5 μl of cell suspension to 2 ml of Na^+-free

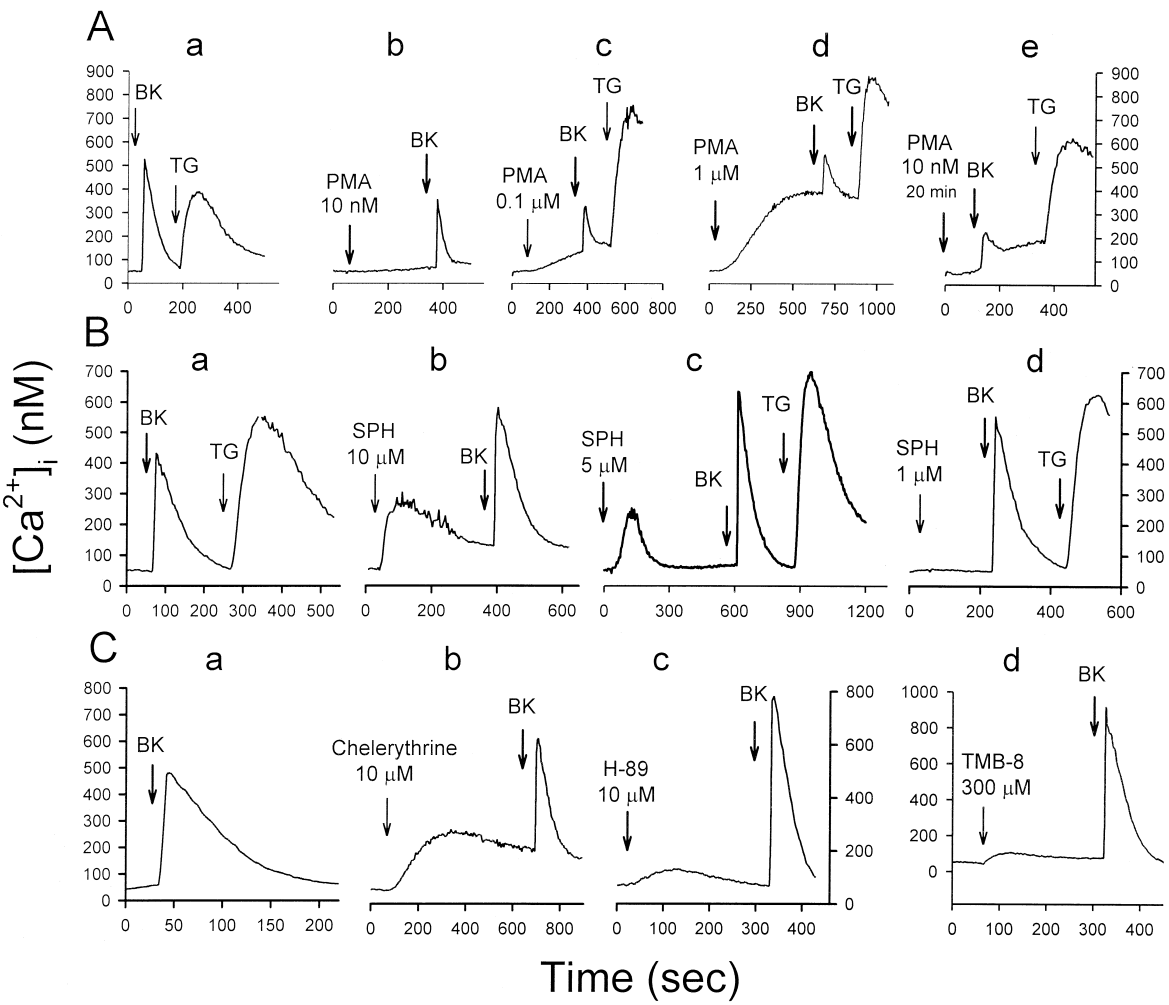

Fig. 5. Effect of a variety of drugs on bradykinin-induced $[Ca^{2+}]_i$ transients in a population of MDCK cells suspended in normal buffer. A, a: Control for b–e; $[Ca^{2+}]_i$ transients induced by bradykinin (BK; 1 μM) and thapsigargin (TG; 0.1 μM). b: Cells were treated with phorbol 12-myristate 13-acetate (PMA; 10 nM) for ~6 min before addition of bradykinin. c–d: Similar to b with phorbol 12-myristate 13-acetate concentration of 0.1 μM and 1 μM, respectively. e: Cells were treated with PMA (10 nM) for 20 min before addition of bradykinin. B, a: Control for b–d; Concentration: bradykinin = 1 μM; thapsigargin = 0.1 μM. SPH = sphingosine. C, a: control for b–d; concentration of bradykinin was 1 μM. All traces are typical of 5–6 experiments.

buffer in a cuvette. This yielded an external $[Na^+]$ of 0.38 mM. This manipulation immediately triggered a rapid and marked $[Ca^{2+}]_i$ transient (peak = 350 ± 20 nM; $n = 5$) which decreased to 150 ± 23 nM in 10 min. This demonstrates the presence of active Na^+/Ca^{2+} exchange. Subsequent addition of bradykinin and thapsigargin induced responses similar to that observed in normal buffer (Fig. 4A; control). Therefore, the role of Na^+/Ca^{2+} exchange in lowering the bradykinin response is negligible.

3.8. Bradykinin elevates $[Ca^{2+}]_i$ by activating phospholipase C

The phospholipase C inhibitor, U73122 (Bleasdale and Fischer, 1993), was applied to test whether bradykinin elevated $[Ca^{2+}]_i$ by activating phospholipase C. Bradykinin (1 μM) added 5 min after U73122 (5–10 μM) pretreatment failed to elevate $[Ca^{2+}]_i$; however thapsigargin elevated $[Ca^{2+}]_i$ normally (not shown; $n = 5$). At 2 μM, U73122 incubation for 5 min only partially inhibited the bradykinin response and at 1 μM U73122 had little effect. U73343, the inactive structural analogue of U73122, was used as a control for the U73122 effect and was found not to affect the bradykinin response (not shown). We also used neomycin, a drug known to reduce IP_3 formation by binding to phosphoinositides (Slivka and Insel, 1988), to see if a decrease in IP_3 levels could affect the bradykinin response. Incubation with neomycin (3 mM) for 20 min reduced the peak amplitude of the bradykinin response by 26 ± 4% (not shown; $n = 5$; $P < 0.05$). Thus, the data indicate that IP_3 accumulation via phospholipase C activation was required for the $[Ca^{2+}]_i$ increase during bradykinin stimulation.

3.9. Modulation of bradykinin-induced $[Ca^{2+}]_i$ transients

The levels of many enzymes and second messengers are known to modulate the bradykinin-induced Ca^{2+} signal and other cellular responses, e.g., protein kinase C (Aboolian et al., 1989; Lou et al., 1992), cyclic AMP-dependent protein kinase A (Kennedy et al., 1995) and cyclic GMP (Harvey and Burgess, 1996). Fig. 5Ab shows that incubation with phorbol 12-myristate 13-acetate (10 nM) for 6 min did not alter resting $[Ca^{2+}]_i$, but reduced the peak amplitude of the bradykinin response by 23% (400 ± 12 nM vs. control = 510 ± 6 nM; $n = 5$; $P < 0.05$), and a longer incubation (20 min; Fig. 5Ae) reduced it to 200 ± 10 nM ($n = 6$) and inhibited the decay. Interestingly, phorbol 12-myristate 13-acetate immediately induced a gradual increase in $[Ca^{2+}]_i$ (Fig. 5Ac) at 0.1 μM, and this effect was enhanced at 1 μM (Fig. 5Ad), and that the bradykinin response was reduced by both doses of phorbol 12-myristate 13-acetate. Importantly, although the bradykinin response was blunted by 10 nM–1 μM phorbol 12-myristate 13-acetate, the thapsigargin-induced $[Ca^{2+}]_i$ transient was dramatically enhanced (by 110% at 1 μM; 820 ± 49 nM vs. 390 ± 38 nM; $n = 5$; $P < 0.05$). Thus, the inhibition by phorbol 12-myristate 13-acetate of the bradykinin response was not due to depletion of internal Ca^{2+} stores. We next tested the effect of the protein kinase C inhibitor sphingosine on the bradykinin response. Fig. 5Bc shows that at 5 μM sphingosine elevated $[Ca^{2+}]_i$ to 220 ± 10 nM ($n = 5$), a response which decayed to baseline in 2.5 min, whereas at 1 μM (Fig. 5Bd) it did not have an effect. At 10 μM (Fig. 5Bb), sphingosine evoked a $[Ca^{2+}]_i$ transient with a similar peak amplitude as that induced by 5 μM; however the decay was much delayed and $[Ca^{2+}]_i$ did not return to baseline in 6 min. After preincubation with sphingosine (1, 5 or 10 μM) for 3–10 min, bradykinin and

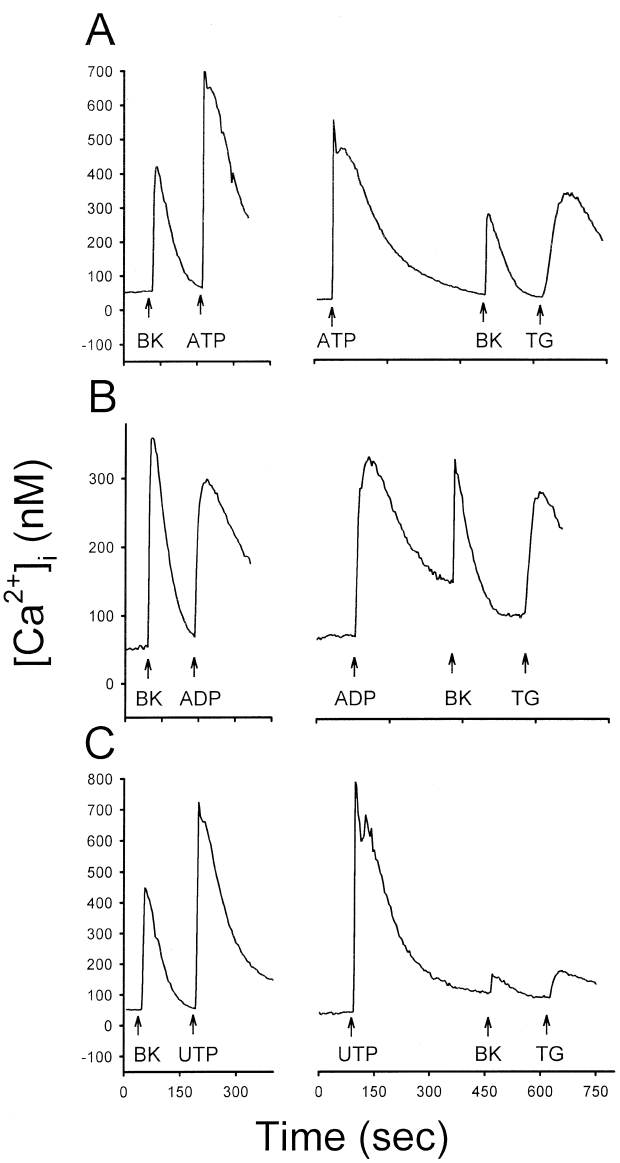

Fig. 6. No heterologous desensitization between bradykinin (BK) and ATP (**A**), ADP (**B**) or UTP (**C**) in a population of MDCK cells suspended in normal buffer. Bradykinin (1 μM) was added before the nucleotide, or vice versa. Concentration: ATP = 10 μM; ADP and UTP = 100 μM; thapsigargin (TG) = 0.1 μM. All traces are typical of 5–6 experiments.

thapsigargin induced $[Ca^{2+}]_i$ transients with similar kinetics as control but the peak values were increased. For instance, incubation with sphingosine (1 μM) for 3 min increased the peak value of the bradykinin response by 34% (550 ± 20 nM vs. 410 ± 15 nM; $n = 5$; $P < 0.05$) and of the thapsigargin response by 17% (610 ± 23 nM vs. 520 ± 13 nM; $n = 6$; $P < 0.05$). Similar to sphingosine, another potent protein kinase C inhibitor chelerythrine induced a slow increase in $[Ca^{2+}]_i$ (Fig. 5Cb; peak = 276 ± 13 nM; $n = 5$) which gradually decreased to ~200 nM in 10 min. Bradykinin added subsequently induced a $[Ca^{2+}]_i$ transient with a peak amplitude 40% higher than control (653 ± 33 nM vs. 466 ± 26 nM; $n = 5$; $P < 0.05$).

H-89, an inhibitor of protein kinase A, was used to examine whether this enzyme played a role in modulating the bradykinin response. Fig. 5Cc shows that H-89 induced a small increase in resting $[Ca^{2+}]_i$ which returned to baseline in 5 min. Bradykinin added subsequently induced $[Ca^{2+}]_i$ increases with a peak 68% higher than control (782 ± 27 nM vs. 466 ± 26 nM; $n = 5$; $P < 0.05$).

TMB-8, which is generally thought of as a Ca^{2+} channel antagonist, was shown to abolish the bradykinin-evoked Ca^{2+} signal in proximal tubule cells without altering the resting $[Ca^{2+}]_i$ (Aboolian and Nord, 1988). In contrast, we found that TMB-8 induced a small, lasting increase in $[Ca^{2+}]_i$ (105 ± 9 nM; $n = 5$; Fig. 5Cd) and increased the peak amplitude of the bradykinin response by 95% (907 ± 38 nM vs. 466 ± 26 nM; $n = 6$; $P < 0.05$) in MDCK cells.

3.10. No heterologous desensitization between bradykinin and ATP, ADP or UTP

We demonstrated homologous desensitization of the bradykinin response in Fig. 2A; however, this desensitization to bradykinin did not greatly affect the stimulation by ATP. To further explore this issue, we performed the experiments depicted in Fig. 6. When applied after the bradykinin response, ATP (or ADP and UTP) induced a robust $[Ca^{2+}]_i$ increase similar to that obtained when the nucleotide was added without prior bradykinin challenge. When added after the nucleotide-induced response, bradykinin and thapsigargin still elevated $[Ca^{2+}]_i$. Bradykinin only induced a small increase in $[Ca^{2+}]_i$ after

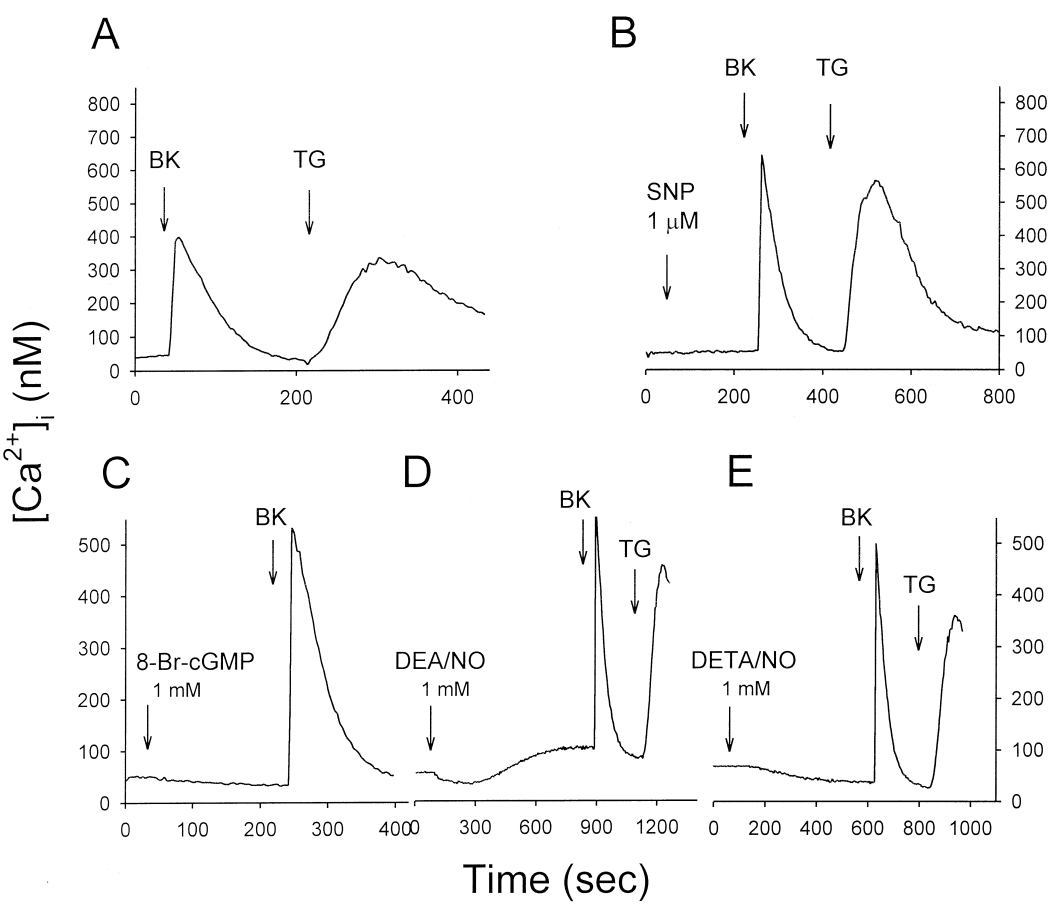

Fig. 7. Effect of increased cytosolic cGMP levels on the bradykinin-induced increase in $[Ca^{2+}]_i$ in a population of MDCK cells suspended in normal buffer. A, control for B–E; concentration was 1 μM for bradykinin (BK) and 0.1 μM for thapsigargin (TG). B–E, cells were pretreated with agents that elevated cGMP levels prior to addition of bradykinin and thapsigargin. SNP: sodium nitroprusside. DEA/NO: diethylamine/nitric oxide. DETA/NO: diethylenetriamine/nitric oxide. All traces are typical of 5–6 experiments.

the UTP-induced response, apparently because UTP had depleted the thapsigargin-sensitive Ca^{2+} store since thapsigargin only induced a small response. This suggests that there was no heterologous desensitization between bradykinin and ATP, ADP or UTP.

3.11. Elevation of cGMP levels enhances the bradykinin response

We next examined whether elevating the cytosolic cGMP level could alter the bradykinin response. The membrane-permeable cGMP analogue, 8-Br-cGMP, slightly lowered resting $[Ca^{2+}]_i$ (Fig. 7C). Subsequent addition of bradykinin induced a $[Ca^{2+}]_i$ transient with a peak amplitude 28% higher than control (510 ± 18 vs. 400 ± 20; $n = 5$; $P < 0.05$). We also examined the effect of three nitric oxide donors: sodium nitroprusside, diethylenetriamine/nitric oxide and diethylamine/nitric oxide. Pretreatment with sodium nitroprusside did not affect resting $[Ca^{2+}]_i$; however, it increased the peak amplitude of the bradykinin response by 72% (Fig. 7B; 686 ± 13 vs. 400 ± 20; $n = 5$; $P < 0.05$). In cells treated with sodium nitroprusside, the peak amplitude of the thapsigargin response was increased by 66% (580 ± 13 vs. 350 ± 18 nM; $n = 5$; $P < 0.05$). Diethylamine/nitric oxide immediately lowered the resting $[Ca^{2+}]_i$ but it gradually rose to ~ 50 nM above the original baseline (Fig. 7D). Bradykinin added subsequently induced a response with a peak amplitude of 555 ± 23 nM (39% over control; $n = 6$; $P < 0.05$). The thapsigargin-induced $[Ca^{2+}]_i$ peak (470 ± 21 nM) was 34% higher than control ($n = 6$; $P < 0.05$). Diethylenetriamine/nitric oxide induced a gradual decrease in basal $[Ca^{2+}]_i$ (Fig. 7E); however, as with diethylamine/nitric oxide, the bradykinin response had a peak amplitude of 500 ± 13 nM, which was 25% higher than control ($n = 6$; $P < 0.05$). The thapsigargin response was not altered by diethylenetriamine/nitric oxide pretreatment ($n = 6$; $P > 0.05$). We next tested whether inhibition of nitric oxide synthase could affect the bradykinin response. Incubation of cells with the nitric oxide synthase inhibitor L-N^G-nitroarginine methyl ester (500 μM, 30 min) did not have an effect (not shown; $n = 5$).

The following drugs did not alter the bradykinin-evoked Ca^{2+} signal: forskolin (100 μM), an adenylyl cyclase activator; isobutylmethylxanthine (300 μM), a phosphodiesterase inhibitor, 8-Br-cAMP (1 mM), a membrane-permeable cAMP analogue, and KN-62 (10 μM), an inhibitor of Ca^{2+}/calmodulin kinase II (not shown).

4. Discussion

In this study we have found that the bradykinin-induced $[Ca^{2+}]_i$ transient in MDCK cells results from a release of Ca^{2+} from the thapsigargin-sensitive Ca^{2+} stores but not from ryanodine-sensitive stores, mitochondria and lysosomes, and that this Ca^{2+} release activates capacitative Ca^{2+} entry. The decay of the bradykinin response might involve Ca^{2+} efflux via plasmalemmal Ca^{2+} pumps and sequestration by the endoplasmic reticulum and/or the nucleus, but not by efflux via Na^+/Ca^{2+} exchange or sequestration by mitochondria or lysosomes.

Our data show that bradykinin induced a maximum Ca^{2+} signal at 10 nM, and at higher doses produced blunted signals. The dose-response relationship resembled closely that reported previously (Pidikiti et al., 1985). We found that bradykinin induced a monophasic $[Ca^{2+}]_i$ transient in MDCK cells, consistent with the previous reports (Lou et al., 1992; Vamos et al., 1995).

Whether or not bradykinin activates Ca^{2+} influx in MDCK cells has been controversial. Lang et al. (1991) found that the bradykinin response was decreased by Ca^{2+} removal whereas a recent report showed that it was not affected by removal or addition of Ca^{2+} (Vamos et al., 1995). We demonstrated that bradykinin activated Ca^{2+} influx on the base of two independent measurements. First, the bradykinin response was reduced by 48% in peak amplitude by Ca^{2+} removal. This reduction could not be due to depletion of internal Ca^{2+} stores because cells were incubated in Ca^{2+}-free buffer for only 1 min. Second, Mn^{2+} quench experiments demonstrated a small but significant Ca^{2+} influx component. We also found that this Ca^{2+} influx resulted from depletion of the thapsigargin-sensitive Ca^{2+} stores because bradykinin failed to elevate $[Ca^{2+}]_i$ when the internal Ca^{2+} stores were depleted by thapsigargin or cyclopiazonic acid. Bradykinin has been reported to trigger Ca^{2+} influx in another renal epithelial cell line (Kitamura and Miller, 1994) and in other non-excitable systems such as endothelial cells (Mendelowitz et al., 1992). This bradykinin-evoked Ca^{2+} influx in MDCK cells is most likely mediated by capacitative Ca^{2+} entry because bradykinin could not induce Ca^{2+} influx after the internal Ca^{2+} stores had been depleted by thapsigargin or cyclopiazonic acid (Fig. 2E and F). This is consistent with the fact that the Ca^{2+} channel blockers we tested did not affect the bradykinin-evoked Ca^{2+} influx. However, the lack of effect of SKF 96365 is somewhat surprising since this compound has been shown to block bradykinin-induced capacitative Ca^{2+} entry in non-excitable as well as excitable cells (Cabello and Schilling, 1993; Lo and Thayer, 1995). We performed two independent experiments to examine the effect of SKF 96365 (1–30 μM) on the bradykinin- and thapsigargin-induced increase in $[Ca^{2+}]_i$: incubation with the blocker for several minutes before addition of bradykinin or thapsigargin in normal buffer; or incubation with the blocker before addition of bradykinin or thapsigargin in Ca^{2+}-free buffer followed by addition of 5 mM Ca^{2+} to induce capacitative Ca^{2+} entry. In neither experiment did we observe an inhibitory effect of SKF 96365 at the concentration range we tested. Recently, Delles et al. (1995) reported that SKF 96365 did not inhibit a thapsigargin-induced inward Ca^{2+} current in

MDCK cells. Thus, it is possible that in MDCK cells bradykinin evokes capacitative Ca^{2+} entry which is insensitive to SKF 96365 at concentrations between 1–30 μM.

Our data confirm a previous report which showed that in MDCK cells bradykinin elevated $[Ca^{2+}]_i$ by activating bradykinin B_2 receptors but not B_1 receptors (Coyne et al., 1989). Moreover, we found that the bradykinin B_2 receptor inhibitor HOE 140 attenuated the bradykinin response, probably by competing for the bradykinin receptor because increasing the bradykinin concentration overcame the HOE 140 inhibition. Another finding was that HOE 140 did not affect the ATP or thapsigargin-induced $[Ca^{2+}]_i$ signal.

Thapsigargin, cyclopiazonic acid and 2,5-di-(*t*-butyl)-1,4-hydroquinone, three inhibitors of the endoplasmic reticulum Ca^{2+} pump, appeared to deplete the same Ca^{2+} store in MDCK cells. The order of potency was thapsigargin > cyclopiazonic acid > 2,5-di-(*t*-butyl)-1,4-hydroquinone. Thapsigargin or cyclopiazonic acid prevented the bradykinin-evoked $[Ca^{2+}]_i$ increase, indicating that the bradykinin-sensitive Ca^{2+} store had been completely depleted by pretreatment with these two inhibitors. However, at 50 μM, 2,5-di-(*t*-butyl)-1,4-hydroquinone could not completely deplete the bradykinin-sensitive stores although it released a similar amount of Ca^{2+} as that released by cyclopiazonic acid. Further, MDCK cells probably lack ryanodine-sensitive Ca^{2+} stores because ryanodine and caffeine failed to elevate $[Ca^{2+}]_i$.

We found that mitochondria and lysosomes contained Ca^{2+} releasable by carbonylcyanide *m*-chlorophynylhydrozone and gly-phe-β-naphthylamide, respectively. The mitochondrial uncoupler carbonylcyanide *m*-chlorophynylhydrozone has been shown to elevate $[Ca^{2+}]_i$ in neuronal cells (Bleakman et al., 1993). However, oligomycin, an inhibitor of the mitochondrial ATP synthase, did not elevate $[Ca^{2+}]_i$. This difference might reflect the different mechanisms of their inhibition of mitochondria. The fact that mitochondria and lysosomes contained releasable Ca^{2+} is consistent with the finding that ionomycin still released a significant amount of Ca^{2+} after depletion of the Ca^{2+} stores sensitive to bradykinin and thapsigargin in the absence of Ca^{2+}. Thus, the internal stores for the bradykinin-induced increase in $[Ca^{2+}]_i$ are the IP_3-coupled, thapsigargin-sensitive stores.

How an agonist-induced Ca^{2+} signal decays in MDCK cells has not been investigated previously. We found that the $[Ca^{2+}]_i$ increase induced by bradykinin might decay partly by efflux via plasmalemmal Ca^{2+} pumps because La^{3+} (100 μM) and extracellular alkalization both slowed the decay of the bradykinin response. Our data showing that efflux might play a key role in the decay of the bradykinin response are similar to those of a study performed with endothelial cells, in which half the total cellular $^{45}Ca_{2+}$ content was shown to leave the cell within 2 min after addition of bradykinin (Cabello and Schilling, 1993). Another important finding in our study was that La^{3+} dramatically slowed the decay of the thapsigargin-induced $[Ca^{2+}]_i$ increase. We interpret this as indicating that, like its effect on the bradykinin response, La^{3+} blocked Ca^{2+} efflux and thus potentiated the thapsigargin response. However, another laboratory has reported that La^{3+} blocked the thapsigargin-induced $[Ca^{2+}]_i$ increase (Delles et al., 1995). We do not know what caused the discrepancy.

We also examined whether Na^+/Ca^{2+} efflux contributed to the decay of the bradykinin response. Lowering extracellular $[Na^+]$ to 0.38 mM elevated $[Ca^{2+}]_i$ significantly, which implies that MDCK cells have an active Na^+/Ca^{2+} exchange as demonstrated previously (Snowdowne and Borle, 1985); however, subsequent increase and decay of bradykinin- and thapsigargin-induced responses were not altered, thus excluding the involvement of Na^+/Ca^{2+} exchange in the decay of the $[Ca^{2+}]_i$ response induced by bradykinin and thapsigargin. The conclusion is supported by our data that amiloride, which could block Na^+/Ca^{2+} exchange, did not affect the bradykinin response.

Decay of the bradykinin response cannot be solely due to return of the mobilized Ca^{2+} to the thapsigargin-sensitive stores (Fig. 3E) because if this were the case, one would expect that thapsigargin added after the bradykinin response would induce an increase in $[Ca^{2+}]_i$ of identical magnitude to that seen without prior bradykinin stimulation. That the thapsigargin response was reduced by ~50% in peak amplitude suggests that part, but not all, of the Ca^{2+} could have returned to the thapsigargin-sensitive stores.

Another explanation for the decay of the bradykinin response is the sequestration of Ca^{2+} by mitochondria and/or lysosomes, since these organelles contain releasable Ca^{2+} under resting conditions. This possibility can be excluded because carbonylcyanide *m*-chlorophynylhydrozone or gly-phe-β-naphthylamide added after the bradykinin response did not release more Ca^{2+} than when they were added to cells prior to bradykinin. Similar results were found for oligomycin. Collectively, our data suggest that the decay of the bradykinin response is partly mediated by efflux via the Ca^{2+} pump and possibly sequestration by the thapsigargin-sensitive stores, and that efflux via Na^+/Ca^{2+} exchange and sequestration by mitochondria and lysosomes are hardly involved. A recent confocal fluorescence microscopy study has provided direct evidence that the nucleus might play a significant role in buffering Ca^{2+} signals evoked by agonists such as bradykinin in smooth muscle cells (Bkaily et al., 1997a) and by thapsigargin in basophilic leukemia cells (Horikoshi et al., 1994). The nucleus also contains IP_3-sensitive receptors that could modulate the nuclear Ca^{2+} concentration (Gerasimenko et al., 1995; Hennager et al., 1995). Thus a possible role of the nucleus in shaping the bradykinin-evoked Ca^{2+} signal in MDCK cells cannot be excluded. The contribution of Ca^{2+} binding proteins was not examined in this study.

Though it is known that IP_3 mediates the bradykinin-induced Ca^{2+} signal in MDCK cells, direct evidence that phospholipase C is involved has not been presented previously. We demonstrated that bradykinin elevated $[Ca^{2+}]_i$ by activating phospholipase C because U73122 (5–10 μM) abolished the bradykinin response while having little effect on the thapsigargin-induced $[Ca^{2+}]_i$ increase. U73122 has also been found to block the ATP-induced $[Ca^{2+}]_i$ increase (Haller et al., 1996; Jan et al., 1998b) and bradykinin-induced phospholipase C activation (Kennedy et al., 1995) in MDCK cells. The observation that neomycin also blunted the bradykinin response further suggests that bradykinin elevated $[Ca^{2+}]_i$ by increasing IP_3 levels.

The bradykinin-induced $[Ca^{2+}]_i$ increase could be modulated by several second messengers. We found that activation of protein kinase C with phorbol 12-myristate 13-acetate blunted the bradykinin response; conversely, inhibition of protein kinase C by sphingosine or chelerythrine potentiated the response, suggesting a role for protein kinase C in the bradykinin response. Previous studies have shown that activation of protein kinase C blunted the bradykinin-induced $[Ca^{2+}]_i$ increase or IP_3 formation in MDCK cells (Pidikiti et al., 1985; Portilla and Morrison, 1986; Aboolian et al., 1989; Coyne et al., 1989) although the underlying mechanism was not clear. Similar to our data, in osteoblasts, phorbol 12-myristate 13-acetate has been found to reduce bradykinin-induced Ca^{2+} influx, and prior treatment with the phorbol 12-myristate 13-acetate inhibitor staurospaurine enhanced the initial internal release and reversed the phorbol 12-myristate 13-acetate effect (Sakai et al., 1992). In addition, we found that phorbol 12-myristate 13-acetate itself induced a significant increase in $[Ca^{2+}]_i$ at doses higher than 0.1 μM by triggering Ca^{2+} influx, but not by depleting the thapsigargin-sensitive stores. Thus, phorbol 12-myristate 13-acetate blunted the bradykinin response presumably by reducing IP_3 accumulation instead of depleting Ca^{2+} stores because the thapsigargin-induced $[Ca^{2+}]_i$ increase, which was independent of IP_3 accumulation, was even enhanced. Phorbol 12-myristate 13-acetate has also been found to activate Ca^{2+} influx in endothelial cells (Murphy et al., 1994). Besides being a protein kinase C inhibitor, sphingosine has been found to mobilize Ca^{2+} in many cell types (Sakano et al., 1996). Our data show that at 10–20 μM, sphingosine induced an increase in $[Ca^{2+}]_i$ which reached a peak amplitude of ~250 nM in 1–2 min whereas at 1 μM it did not have an effect. At 1–10 μM, sphingosine pretreatment for 3–10 min significantly enhanced the bradykinin response. Interestingly, we found that the $[Ca^{2+}]_i$ increase induced by thapsigargin added after the bradykinin response was also enhanced. The observation that this potentiating effect of sphingosine on the bradykinin response was mimicked by another protein kinase C inhibitor chelerythrine suggests that inhibition of protein kinase C could augment the bradykinin-induced $[Ca^{2+}]_i$ increase, probably by increasing Ca^{2+} influx instead of releasing internal Ca^{2+} because the $[Ca^{2+}]_i$ increase induced by subsequently added thapsigargin was also enhanced.

Bradykinin has been reported to increase cytosolic cAMP levels in MDCK cells (Hassid, 1983). In MDCK-D1 cells, a subclone of MDCK cells, elevation of cAMP levels was found to blunt bradykinin-induced phospholipase C activity and arachidonic acid release, an effect which could be reversed by H-89 (Kennedy et al., 1995). However, our data show that forskolin, isobutylmethylxanthine, and 8-Br-cAMP, agents that elevate cAMP levels, all failed to affect resting $[Ca^{2+}]_i$ and the bradykinin response whereas H-89 significantly enhanced them. Thus, it appears that bradykinin-induced $[Ca^{2+}]_i$ transients are not altered by activation of protein kinase A via cAMP but can be enhanced when protein kinase A is inhibited. Furthermore, KN-62 did not affect the bradykinin response, suggesting that Ca^{2+}/calmodulin-dependent protein kinase II might not be involved. Our data showing that an increase in cAMP levels did not alter resting $[Ca^{2+}]_i$ is consistent with a previous report which showed that forskolin and isoproterenol both elevated cAMP levels but did not affect $[Ca^{2+}]_i$ in MDCK cells (Kurstjens et al., 1990). However, in an earlier study, isoproterenol, forskolin, isobutylmethylxanthine and 8-Br-cAMP were found to elevate $[Ca^{2+}]_i$ significantly (Chase and Wong, 1988). The reason for this difference is unclear.

TMB-8 was found to block the bradykinin-induced $[Ca^{2+}]_i$ increase in proximal tubular cells (Aboolian and Nord, 1988) and the prostaglandin-induced $[Ca^{2+}]_i$ increase in MDCK cells (Aboolian et al., 1989) without altering resting $[Ca^{2+}]_i$. In contrast, we found that TMB-8 (100–300 μM) induced a small but significant increase in $[Ca^{2+}]_i$ and augmented the subsequent bradykinin-induced increase in $[Ca^{2+}]_i$. This is unlikely to be an artifact because TMB-8 did not interfere with fura-2 fluorescence and similar results were obtained with TMB-8 purchased from two different sources (Sigma and RBI). The observation that in TMB-8-treated cells, the $[Ca^{2+}]_i$ transient induced by thapsigargin (added after bradykinin) was inhibited suggests that TMB-8 might enhance the ability of bradykinin to release Ca^{2+} from thapsigargin-sensitive stores via an unknown mechanism (Jan, unpublished data).

Pretreatment with bradykinin did not attenuate the $[Ca^{2+}]_i$ response to subsequent addition of ATP (or ADP and UTP); and vice versa, pretreatment with ATP (or ADP and UTP) did not attenuate the $[Ca^{2+}]_i$ response to subsequent addition of bradykinin. This suggests that there was no heterologous desensitization between bradykinin and these nucleotides. There was also no heterologous desensitization between bradykinin and prostaglandins (Aboolian et al., 1989) or charbachol (Lou et al., 1992) in MDCK cells.

Bradykinin induces renal vasodilation by increasing the synthesis of nitric oxide thus increasing cGMP levels, leading to diuresis and natriuresis (Backmann and Mundel, 1994). Therefore, it is of interest to know whether changes

in cGMP levels could modulate the bradykinin response. We found that sodium nitroprusside, diethylamine/nitric oxide, diethylenetriamine/nitric oxide and 8-Br-cGMP, drugs known to increase cGMP levels, consistently potentiated the bradykinin response; however, blocking nitric oxide synthase with L-N^G-nitroarginine methyl ester had no effect. It appears that increasing the cGMP level could potentiate the bradykinin response, probably by increasing Ca^{2+} influx since the thapsigargin response was also enhanced by sodium nitroprusside and diethylamine/nitric oxide; however, nitric oxide release is not required for bradykinin's actions. An elevation of the cGMP level has also been shown to potentiate bradykinin-induced increases in $[Ca^{2+}]_i$ in tracheal epithelium (Sakai et al., 1995); however, the opposite results were found in PC12 cells (Clementi et al., 1995). Thus, cGMP might play a stimulatory or inhibitory role in the bradykinin response depending on the cell type.

To conclude, in a study of Ca^{2+} homeostasis in MDCK cells, we found that bradykinin elevated $[Ca^{2+}]_i$ by activating bradykinin B_2 receptors and phospholipase C, resulting in accumulation of IP_3 and a subsequent release of Ca^{2+} from thapsigargin-sensitive internal Ca^{2+} stores. Mobilization of internal Ca^{2+} in turn activated capacitative Ca^{2+} entry. The decay of the Ca^{2+} signal might involve the efflux of Ca^{2+} via plasmalemmal Ca^{2+} pumps and its return to stores in the endoplasmic reticulum and/or possibly the nucleus, but not by efflux via Na^+/Ca^{2+} exchange or sequestration by mitochondria and lysosomes. Several second messengers could modulate the bradykinin response such as protein kinase C, protein kinase A, and cGMP.

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