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Intracellular Ca^{2+} depletion and Ca^{2+} channel blockers increase renal kallikrein secretion

Guy Bompart, Ivan Tack, Jean-Loup Bascands, Jean-Pierre Girolami *

Institut National de la Santé et de la Recherche Médicale, U388, Institut Louis Bugnard, CHU Rangueil, 31054 Toulouse, France

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Abstract

This study examined the effect of various manipulations of intracellular Ca^{2+} on kallikrein release by renal cortical slices. Increasing the extracellular Ca^{2+} concentration and the addition of Ca^{2+} ionophore A23187 was without effect on kallikrein release. In contrast, kallikrein release was enhanced by the addition of either extracellular or intracellular Ca^{2+} chelators in Ca^{2+}-free medium and by two Ca^{2+} channel blockers, verapamil and nifedipine. Kallikrein release was also highly enhanced in depolarising medium (10–100 mM potassium chloride). Since potassium chloride induced a dose-related increase in free cytosolic Ca^{2+} which was abolished by nifedipine whereas the stimulation of kallikrein secretion persisted, a direct stimulating effect of potassium, at least at sub-physiological concentration, is suggested. Similarily, inhibition of either sodium/potassium-ATPase and Ca^{2+}ATPase by ouabain and vanadium respectively, was also without effect on kallikrein secretion. Taken together, these results indicate that intracellular Ca^{2+} depletion, Ca^{2+} channel blockers and high extracellular K^+ concentrations, acting through different mechanisms, are effective stimuli for kallikrein secretion, at least in the isolated renal cortical slice.

Keywords: Renal kallikrein secretion; Ca^{2+}; K^+; Ca^{2+} channel antagonist; Ouabain; Vanadium

1. Introduction

The renal kallikrein-kinin system is an enzymatic system participating in both complex intrarenal and extrarenal events such as control of water and electrolyte excretion, renal vascular resistance, renin release and regulation of blood pressure (Mayfield and Margolius, 1983; Johnston et al., 1984; Scicli and Carretero, 1986; Carretero and Scicli, 1990). Many in vivo studies show close relationships between the renin-angiotensin system and the kallikrein-kinin system. This viewpoint is consistent with the anatomic localization of these two enzymatic systems (i.e the connecting tubule-collecting duct and the juxtaglomerular apparatus-connecting tubule) proposed by Vio et al. (1992). It is suggested that the renin-angiotensin and kallikrein-kinin systems may act in opposite manners to control blood pressure (Carretero and Scicli, 1990; Vio et al., 1992). Therefore, the control of the biosynthesis and secretion of these two systems remains a crucial point to be considered. The control of renin release has stimulated a large number of studies and there is a general agreement to consider that Ca^{2+} influx is an inhibitory signal for renal renin release (Park et al., 1981; Churchill, 1979, 1990; Churchill and Churchill, 1980, 1982; Churchill et al., 1983; Nakamura et al., 1989). On the other hand, little information is available regarding the effect of Ca^{2+} on renal kallikrein release.

In view of these studies, we now attempted to test the hypothesis that a Ca^{2+}-linked mechanism could participate in kallikrein secretion. Since no isolated renal cellular system is available for studying renal kallikrein secretion, cortical kidney slices were used because they contain the kallikrein-producing cells that synthesize and release active and inactive kallikrein (Nustad and Vaaje, 1975; Beasley et al., 1987; Girolami et al., 1990a,b; Vio et al., 1992). Moreover, this in vitro model eliminates changes in perfusion pressure that occur in vivo and influence the status of the renal kallikrein-kinin system.

* Corresponding author. INSERM U388, Institut Louis Bugnard, CHU Rangueil, 31054 Toulouse, France. Tel. (33) 61 32 26 21, fax (33) 62 17 25 54.

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2. Materials and methods

2.1. Preparation and incubation of renal cortical slices

Renal cortical slices were prepared using a modification (Girolami et al., 1990a,b) of the method currently used for renin secretion studies (Park et al., 1981; Churchill, 1979, 1990; Churchill and Churchill, 1980, 1982; Churchill et al., 1983; Nakamura et al., 1989). In each experiment 4–6 male Sprague-Dawley rats (Ifa Credo) weighing 300–350 g with free access to food (UAR A40, normal sodium: 113 mEq/kg) and tap water were used. The rats were killed by decapitation, the kidneys were quickly removed, decapsulated and placed in ice-cold saline solution. The renal cortex was cut with a razor blade and then sliced approximately 0.3 mm thick with a tissue slicer (TC-2 Sorvall). The slices were pooled and washed twice with 40 ml fresh Krebs-Ringer solution (KRS) to remove small tissue debris. After this washing period, approximately 100–150 mg of the slices were incubated in a flask containing 2 ml of prewarmed KRS in a water bath at 37° C for 30 min under a continuous stream of 95% O_2, 5% CO_2. Except for the modifications described in the Results section, the composition of the incubation medium was (in mM): 125 NaCl, 4 KCl, 2.6 $CaCl_2$, 1.2 NaH_2PO_4, 0.8 $MgSO_4$, 25 $NaHCO_3$, 10 glucose, and pH 7.4. At the end of the incubation period in all the experimental protocols the incubation medium was drawn up and centrifuged 5 min at 4000 rpm at 4° C. The slices were weighed and the supernatant was stored at −30° C until kallikrein measurements were performed.

2.2. Kallikrein determination

The immunoreactive concentration of kallikrein was measured with a specific radioimmunoassay (Girolami et al., 1987) using rabbit antiserum raised against purified rat urinary kallikrein. In this previous study, we had demonstrated that the antibody binds both the active and inactive forms of kallikrein since trypsinisation of the samples did not induce any changes in the immunoreactive concentration of kallikrein. The results were calculated using the Logit-Log linearization of the standard curve and are expressed in nanograms of immunoreactive kallikrein released per 100 mg of tissue during the 30 min of incubation (ng/100 mg tissue/30 min).

2.3. Calcium concentration measurements

Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was measured in rat cortex fragments using the fura-2 method as now currently performed in the laboratory (Bascands et al., 1994). Briefly, the cortex fragment was loaded with fura-2/AM (5 μM final concentration, at 37° C for 15–30 min), rinsed with the perfusing solution which was Krebs-Ringer of the following composition (in mM): 10 Hepes, 145 NaCl, 2.5 KH_2PO_4, 1 $CaCl_2$, 1 $MgSO_4$, 1% bovine serum albumine (fatty acid free) and 10 glucose. The cortex fragment was then transferred onto a glass coverslip coated with collagen to which the cortex fragment adhered. The slide was fixed at the bottom of a superfusion device as described in detail by Taniguchi et al. (1989) and continuously superfused at a rate of 0.4 ml/min with the desired solution kept at 37° C. The preparation was adjusted in the window of a Nikon fluorescent microscope and fluorescence measurements were made with a Spex Flurilog spectroflurometer set for alternative dual-wavelength excitation at 336 and 384 nm respectively. The fluorescence emitted at 505 nm was collected by a computerized photomultiplier which averaged the emission collected over a 0.5-s period at each excitation wavelength. Autofluorescence of each preparation at each excitation wavelenght was automatically subtracted. The $[Ca^{2+}]_i$ was calculated from the equation of Grynkiewicz et al. (1985): $[Ca^{2+}]_i = K_D \times (R - R_{min}/R_{max} - R) \times \lambda$, where K_D (= 224 nM) is the dissociation constant of the complex [fura 2-Ca^{2+}] and R_{min}, R_{max} and λ are constant parameters that depend

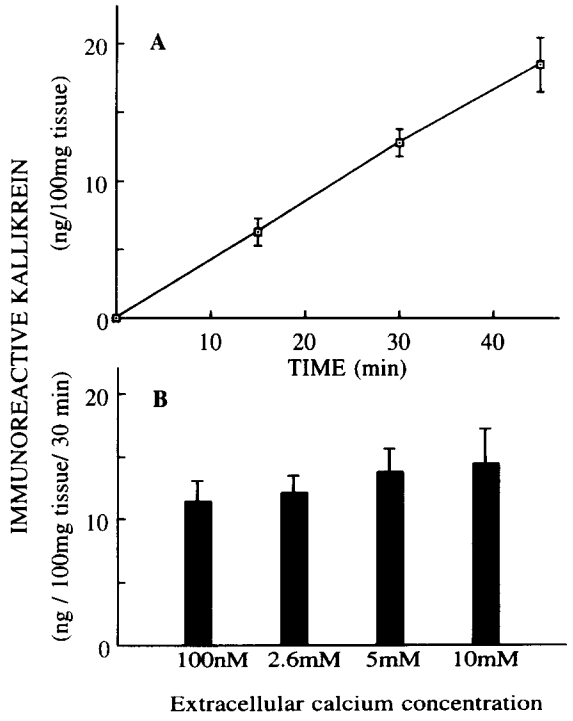

Fig. 1. (A) Kinetics of immunoreactive kallikrein release. Freshly prepared rat kidney slices were incubated for increasing periods of time from 15 to 45 min. (B) Effect of increasing concentrations of Ca^{2+} on kallikrein release. Kallikrein was measured by radioimmunoassay using an antibody against purified rat urinary kallikrein. Each point represents the mean ± S.D. of six separate observations at each incubation time.

on the optical system used. For our experimental conditions they were $R_{min} = 0.9$, $R_{max} = 18$ and $\lambda = 4$.

The data are expressed as mean values ± S.D. and were analysed by unpaired Student's t-test. In all comparisons, differences were considered significant at $P < 0.05$.

2.4. Drugs and abbreviations

Ouabain, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), Ca^{2+} ionophore A23187, bovine serum albumin, nifedipine and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA) and bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)-ester (BAPTA/AM) were purchased from Calbiochem Co. (San Diego, CA, USA). Fura-2 was from Molecular Probe. All other reagents were provided by Merck (Darmstadt, Germany).

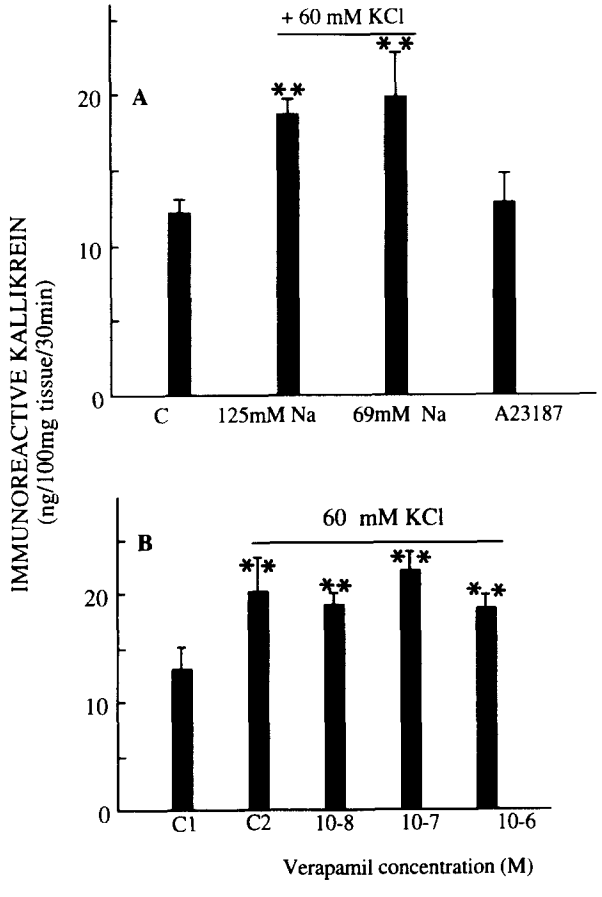

Fig. 2. (A) Effect of depolarizing medium (60 mM K^+ either in 125 or in 69 mM Na^+) and Ca ionophore A23187 on kallikrein secretion by rat kidney slices. C: control slices incubated in normal medium (2.6 mM Ca^{2+}). Freshly prepared kidney slices were incubated for 30 min in the appropriate medium. Kallikrein was measured by radioimmunoassay using an antibody against purified rat urinary kallikrein. Each bar represents the mean ± S.D. of six separate observations. $^{**}P < 0.01$ when compared to control C. (B) Effect of increasing concentrations of verapamil on kallikrein secretion rate of depolarized rat kidney slices incubated in depolarizing medium. C1: control in normal medium. C2: control in depolarizing medium (60 mM K^+). Each bar represents the mean ± S.D. for six separate observations. $^{**}P < 0.01$ when compared to control C1.

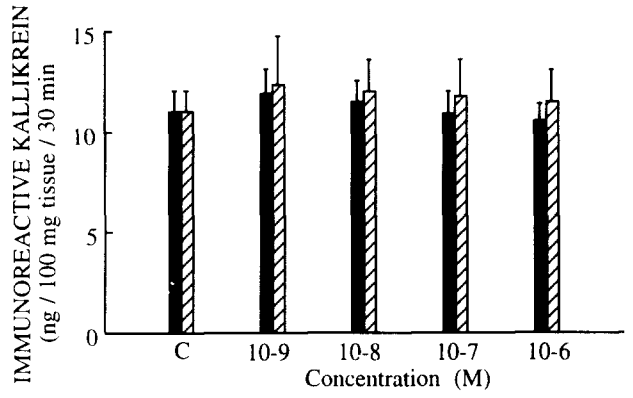

Fig. 3. Effect of increasing concentrations of ouabain (black bar) and vanadium (hatched bar) on kallikrein secretion by rat kidney slices. C: control slices incubated in normal medium (2.6 mM Ca^{2+}). Freshly prepared kidney slices were incubated for 30 min in the appropriate medium. Kallikrein was measured by radioimmunoassay using an antibody against purified rat urinary kallikrein. Each bar represents the mean ± S.D. of six separate observations.

3. Results

3.1. Kallikrein release under basal conditions

This preliminary protocol was carried out to ascertain that kallikrein secretion was time-dependent. As shown in Fig. 1A, kallikrein release in the incubation medium was linear in a time-dependent fashion at least for 45 min. The secretion rate of immunoreactive kallikrein was 12.7 ± 0.7 ng/100 mg tissue/30 min. Furthermore, the recovery of purified kallikrein added to the incubation medium (with or without renal slices) was 85 ± 12%, indicating minor degradation in the absence of protease inhibitors.

3.2. Effect of increasing extracellular Ca^{2+} and membrane depolarization

Incubation of renal cortical slices in medium containing increasing Ca^{2+} concentrations from very low Ca^{2+}, 100 nM to 10 mM, did not modify kallikrein release (Fig. 1B). The Ca^{2+} ionophore A23187 (10^{-6} M), which permeabilises the plasma membrane and facilitates Ca^{2+} influx, did not change kallikrein secretion (Fig. 2A) when added to control medium (2.6 mM

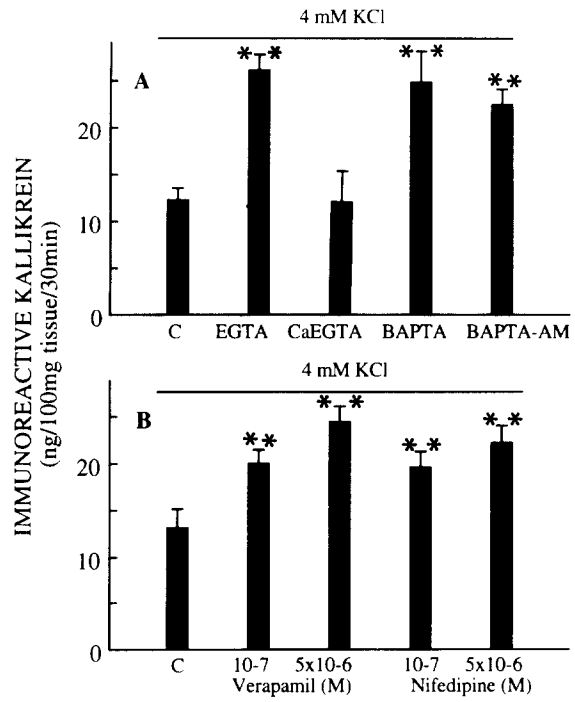

Fig. 4. (A) Effect of EGTA, CaEGTA, BAPTA, and BAPTA-AM on kallikrein secretion by rat kidney slices. C: control slice incubated in normal medium (2.6 mM Ca^{2+}); EGTA: Ca-free medium + 2 mM Na_2EGTA; CaEGTA: 2.6 mM $CaCl_2$ + 2 mM CaEGTA; BAPTA: Ca^{2+}-free medium + 2 mM BAPTA; BAPTA-AM: Ca^{2+}-free medium + 0.1 μM BAPTA-AM. Each bar represents the mean ± S.D. of six separate observations. $^{**}P < 0.01$ when compared to control. (B) Effect of verapamil and nifedipine on kallikrein release. C: control in normal medium (2.6 mM Ca^{2+}). Freshly prepared kidney slices were incubated for 30 min in the appropriate medium. Kallikrein was measured by radioimmunoassay using an antibody against purified rat urinary kallikrein. Each bar represents the mean ± S.D. for six separate observations. $^{**}P < 0.01$ when compared to control.

Ca^{2+}). On the other hand, K^+ depolarisation obtained with either 60 mM KCl added to the normal medium or with 60 mM KCl added to medium from which 60 mM NaCl had been omitted so that osmolality was held constant, significantly enhanced kallikrein secretion by 54 and 64% respectively, when compared to the control (Fig. 2A). In view of determining if this stimulatory effect was due to direct potassium influx or to a Ca^{2+} influx caused by the opening of voltage-dependent Ca^{2+} channels, slices were incubated for 30 min in 60 mM K^+ medium in the presence of increasing concentrations of verapamil, from 10^{-8} to 10^{-6} M. As shown in Fig. 2B, increasing the verapamil concentrations from 10^{-8} to 10^{-6} M, in depolarizing medium, did not significantly modify the stimulated kallikrein secretion rate. Thus, verapamil did not antagonize the stimulatory effect of K^+ depolarisation on kallikrein secretion. The same pattern (results not shown) was observed with another Ca^{2+} channel blocker, nifedipine. When slices were incubated for 30 min in medium containing 10^{-9} to 10^{-6} M ouabain (Fig. 3) or pentavalent *o*-vanadium (NaO_3V, Fig. 3) kallikrein secretion was unchanged by these two compounds which are presumed to increase intracellular Ca^{2+}.

3.3. Effect of decreasing intracellular Ca^{2+}

Three different Ca^{2+}-containing media were used: (i) the basal medium (defined as control) contained 2.6 mM $CaCl_2$, (ii) the CaEGTA medium contained 2.6 mM $CaCl_2$ plus 2 mM CaEGTA, (iii) the Ca^{2+}-free medium contained 2 mM Na_2EGTA. As shown in Fig. 4A, in CaEGTA medium, kallikrein secretion was not affected (11.7 ± 1.6 versus 12.1 ± 1.4 ng/100 mg tissue/30 min) but was significantly increased, by 116% (26.1 ± 1.6 versus 12.1 ± 1.4 ng/100 mg tissue/30 min) in Ca^{2+}-free medium when compared to the control. Since media of both types contained 2 mM EGTA, the difference in rates can be attributed specifically to the difference in Ca^{2+} concentrations. Another extracellular Ca^{2+} chelator, BAPTA, also significantly stimulated kallikrein secretion by 105% when added at the same concentration (2 mM) to Ca^{2+}-free medium (Fig. 4A). Prior addition (15 min) of 10^{-7} M BAPTA-AM, an intracellular Ca^{2+} chelator, to Ca^{2+}-free medium, significantly increased the basal kallikrein secretion rate by 85% (Fig. 4A). It should be noted that, in non-depolarizing medium (4 mM K^+), verapamil (10^{-7}

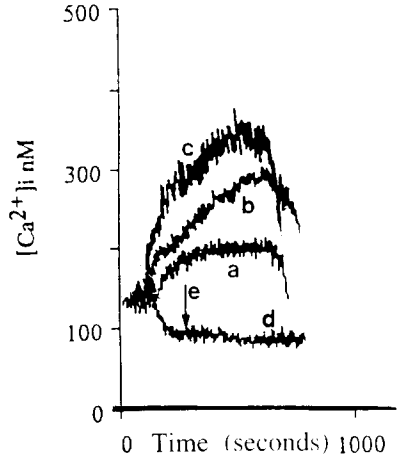

Fig. 5. Effect of increasing concentrations of potassium chloride (KCl) on free cytosolic calcium concentration ($[Ca^{2+}]_i$): (a) KCl 10 mM; (b) KCl 30 mM; (c) KCl 60 and 100 mM (no difference), (d) nifedipine (0.5 μM); (e) arrow indicates KCl superfusion in the presence of nifedipine (0.5 μM). Cortex fragments previously loaded with fura-2 were attached onto a coverslip, superfused with the test solution for about 5 min and the emitted fluorescence was continuously recorded through a Nikon fluorescent microscope as indicated in the Materials and methods section. After computerized calculation the fluorescence was converted into Ca^{2+} concentration expressed as nM. Resting $[Ca^{2+}]_i$ varied in the range of 129 ± 18 nM. These patterns are representative of at least four different experiments and the statistical analysis of similar patterns obtained from at least four different experiments is summarized in Table 1.

or 0.5×10^{-6} M) significantly increased kallikrein secretion by 53 or 88% (Fig. 4B) In the same non-depolarizing medium, nifedipine (10^{-7} or 0.5×10^{-6} M) also significantly increased kallikrein secretion by 50 and 70% (Fig. 4B).

3.4. Relationships between changes in free cytosolic Ca^{2+} and kallikrein secretion

As shown in Fig. 5, increasing the KCl concentrations in the extracellular medium from 10 to 100 nM induced a dose-related increase in $[Ca^{2+}]_i$ which is summarized in Table 1. However, a rise in free cytosolic Ca^{2+} was achieved with a K^+ concentration lower than that required to induce a minimal significant increase in kallikrein secretion. Furthermore the effect of 60 mM K^+ on the increase of $[Ca^{2+}]_i$ was blocked by nifedipine (Fig. 5) but not by verapamil. On the other hand, nifedipine inhibited the K^+-induced rise in free cytosolic Ca^{2+} but did not reduce the stimulating effect of K^+ on kallikrein release. Furthermore nifedipine alone, but not verapamil, reduced the resting $[Ca^{2+}]_i$ level. Finally, verapamil and nifedipine increased kallikrein secretion.

4. Discussion

Using an in vitro renal model, we investigated kallikrein secretion while manipulating Ca^{2+} homeostasis. This in vitro model allowed the study of kallikrein secretion independently of the effect of change in perfusion presure which is a well established stimulus for renal kallikrein release (Misumi et al., 1983). Furthermore, since renal kallikrein synthesis occurs mainly in the connecting cells of the distal tubule, and since to our knowledge it is not possible to obtain such isolated cells, the cortical kidney slice appears the best compromise to study direct actions on renal kallikrein release and the slice model has been successfully used by different groups, not only to study kallikrein release (Nustad and Vaaje, 1975; Girolami et al., 1990a,b), but also to investigate renin secretion (Park et al., 1981; Churchill, 1979, 1990; Churchill and Churchill, 1980, 1982; Churchill et al., 1983; Nakamura et al., 1989).

Increasing the extracellular Ca^{2+} concentration over extra-physiological ranges slightly stimulated kallikrein secretion by 13 and 19% respectively when compared to the kallikrein secretion rate under normal extracellular Ca^{2+} concentrations. This insignificant effect may be attributed to a non-physiological mechanism induced by supra-elevated Ca^{2+} concentrations. Studying the effect of norepinephrine on the release of kallikrein-like esterase from rat submandibular gland cells, Izumi (1984) showed similarily that such supra-elevated Ca^{2+} concentrations (9 mM) also slightly increase kallikrein secretion when compared to normal extracellular Ca^{2+} concentrations. Increasing free cytosolic Ca^{2+} concentrations with Ca^{2+} ionophores, such as A23187, which are thought to facilitate the bidirectional fluxes of Ca^{2+} across the cell membrane (Harada et al., 1979), also had no effect on kallikrein secretion in normal-Ca^{2+}extracellular fluid (2.6 mM). Vanadate inhibition of either Na,K-ATPase or Ca^{2+}-ATPase activities (Churchill and Churchill, 1980) and ouabain inhibition of Na,K-ATPase activity (Churchill, 1979; Akeda and Brody, 1978) would be expected to increase intracellular Ca^{2+} and therefore would be expected to be without any effect as demonstrated by our results.

In the absence of Ca^{2+} in the incubation medium ($Ca^{2+} = 100$ nM which was near the resting intracellular Ca^{2+}, as complete extracellular Ca^{2+}-free medium is a non-steady state) kallikrein secretion was unchanged. In contrast, kallikrein secretion was increased when Na_2EGTA (2 mM) was added to the same medium, lowering Ca^{2+} from 100 nM to < 10 nM. Since CaEGTA (2 mM) added to the control medium (2.6 mM) was without effect, this stimulatory effect on kallikrein secretion was specifically attributed

Table 1
Dose-dependent effect of potassium chloride on free cytosolic Ca^{2+} and on immunoreactive kallikrein secretion

Addition in incubation medium	Free cytosolic Ca^{2+} (nM)		Kallikrein secretion (ng/100 mg tissue/30 min)	
None	129 ± 18	(12)	12.8 ± 2.7	(10)
+ 10 mM K^+	189 ± 21 [b]	(4)	13.8 ± 3.4	(6)
+ 30 mM K^+	293 ± 29 [b]	(4)	17.1 ± 3.2 [a]	(6)
+ 60 mM K^+	352 ± 32 [b]	(8)	21.5 ± 4.2 [b]	(6)
+ 100 mM K^+	334 ± 38 [b]	(8)	22.8 ± 3.9 [b]	(6)
Verapamil (0.5 μM)	118 ± 18	(4)	24.8 ± 3.3 [b]	(6)
Nifedipine (0.5 μM)	84 ± 11 [b]	(6)	23.3 ± 4.1 [b]	(6)
+ 60 mM K^+ + nifedipine	138 ± 32	(8)	23.5 ± 5.2 [b]	(6)
+ 60 mM K^+ + verapamil	322 ± 32 [b]	(8)	22.8 ± 4.7 [b]	(6)

Data for Ca^{2+} concentration are maximum values taken at the peak of the Ca^{2+} increase as shown in Fig. 5 and are expressed as means ± S.D. The number of experiments is in parentheses. Key: K^+, potassium chloride. [a] $P < 0.05$ and [b] $P < 0.01$ when compared to values obtained in the absence of KCl.

to the absence of extracellular ionized Ca^{2+}. Moreover, another Ca^{2+} chelator, BAPTA (2 mM), and BAPTA-AM (10^{-7} M), an intracellular Ca^{2+} chelator, also increased kallikrein secretion by 105 and 85% respectively when added to Ca^{2+}-free medium. These findings suggest strongly that the rise in kallikrein secretion may be mediated by a depletion of intracellular free Ca^{2+}. In addition, membrane depolarisation achieved with increasing concentrations of K^+ was associated with a dose-dependent rise in free cytosolic Ca^{2+} induced kallikrein secretion. However, we found in the present paper that the increase in free cytosolic Ca^{2+} has no effect on kallikrein secretion, thus the stimulating effect of K^+ is due to K^+ itself and not to a Ca^{2+} rise resulting from opening of voltage-operated Ca^{2+} channels by K^+ as previously reported for the same slice model (Churchill and Churchill, 1982; Churchill, 1990). This assumption is confirmed by the dissociating effect of nifedipine which blocks the K^+-induced Ca^{2+} rise but not the stimulating effect of K^+ on kallikrein secretion. Therefore stimulating effects of K^+ and Ca^{2+} are mediated through two different pathways. In this respect, Vio and Figueroa (1987) showed that rats fed with a high K^+ diet had higher values of urinary kallikrein excretion and presented hypertrophy and hyperplasia of the kallikrein-containing cells. The ultrastructural changes and the enhanced kallikrein urinary excretion led these authors to hypothesize that the high K^+ diet increased kallikrein synthesis and secretion.

On the other hand, verapamil and nifedipine also increased kallikrein secretion. The stimulating effect of Ca^{2+} channel blockers is consistent with the normalization of urinary kallikrein excretion previously reported (Tsunoda et al., 1986) in patients with essential hypertension after nifedipine treatment. Additionally, an increase in urinary kallikrein excretion was demonstrated in hypertensive patients receiving nifedipine (Madeddu et al., 1987) but the authors suggested that this might be a consequence rather than the cause of renal effects induced by Ca^{2+} antagonists. However, the acute stimulating effect of Ca^{2+} channel blockers, reported in the present study, is specially worth pointing out. It can be reasonably postulated that an increase in kallikrein secretion could be associated to high kinin formation capacity. Since kinins are potent natriuretic and vasodilator peptides, the present observation may indicate a potential mechanism to account for the beneficial effect of Ca^{2+} channel blocker administration. Although the present results were obtained with renal tissue, similar effects can be expected in the systemic circulation because the presence of a kallikrein-like enzyme has been identified in vascular walls (Nolly et al., 1990). If such a stimulating effect of Ca^{2+} channel blockers on kallikrein secretion is confirmed for vascular walls, this observation could provide evidence for a possible participation of the parietal kallikrein-kinin system in blood pressure regulation.

In conclusion, we bring new direct evidence that a decrease in intracellular Ca^{2+}, Ca^{2+} channel blockers and high extracellular K^+ concentrations stimulate kallikrein secretion. Indeed, the two stimuli act via two independent mechanisms. Whereas when we used K^+, we expected to manipulate Ca^{2+} concentration only, we defined a new situation where kallikrein release is increased. The present data indicate a possible control of kallikrein exocytosis by K^+ and Ca^{2+}.

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