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VASOPRESSOR PEPTIDES AND DEPOLARIZATION STIMULATED Ca^{2+}-ENTRY INTO CULTURED VASCULAR SMOOTH MUSCLE

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SUMMARY ^{45}Ca-uptake was measured in monolayers of cultured rat aortic smooth muscle cells. Sufficient extracellular ^{45}Ca could be removed by a 90 second cold La^{3+} wash to reveal stimulation of ^{45}Ca-uptake by high K^+-depolarization and the vasopressor peptides angiotensin II and vasopressin. The high K^+-stimulated ^{45}Ca-influx was blocked by a dihydropyridine-type Ca^{2+}-antagonist while that stimulated by angiotensin II or vasopressin was not. The ^{45}Ca-influx stimulated by high K^+- depolarization was additive to that stimulated by angiotensin II. Vasopressin and angiotensin II stimulated ^{45}Ca-fluxes were not additive. It is concluded that vasopressor peptides stimulate Ca^{2+}-entry through receptor operated Ca^{2+}-channels which are distinct from voltage gated Ca^{2+}-channels.

INTRODUCTION The classification of separate receptor operated- and voltage gated Ca^{2+}-channels was based on distinct pharmacological and electrical properties of agonist and depolarization stimulated Ca^{2+}-influx into smooth muscle cells (1,2,3).

The advent of the giga seal patch clamp technique quickly revealed several types of voltage gated Ca^{2+}-channels in nerve, cardiac and smooth muscle (4,5,6,7) while only very recently has it been possible to record unitary currents from receptor operated Ca^{2+}-channels in neutrophils (8), smooth muscle (9), platelets (10) and endothelium (11).

The receptor operated Ca^{2+}-channels are distinguished from the voltage gated ones mainly by their insensitivity to both changes in voltage and applications of dihydropyridine calcium antagonists and their lower Ca^{2+}-selectivity. The latter property in addition to others, such as, the necessity to block K^+-

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channels make it difficult to measure Ca^{2+}-currents through these channels under physiological conditions. On the other hand, there also exist some difficulties with the quantitation of agonist stimulated ^{45}Ca-influx in smooth muscle tissues due to the requirement of cold washing procedures for the removal of extracellular ^{45}Ca. For this latter reason it should prove advantageous to use monolayers of cultured smooth muscle cells in which all cells can be directly exposed to the experimental solutions.

A previous study on a line of cultured smooth muscle cells was successful in measuring dihydropyridine sensitive ^{45}Ca-uptake in response to high K^+ depolarization (12). However, its visualization required lowering the $[Ca^{2+}]_e$ to 0.1 mM and pretreatment with EGTA. In this study we measured both agonist and depolarization stimulated ^{45}Ca-influx at normal calcium concentration.

METHODS

Smooth muscle cell culture Aortic smooth muscle cells were isolated from spontaneously hypertensive rats by enzymatic disaggregation of medial tissue as described by Chamley-Campbell et al (13) and characterized as described previously (14). Cultures were used between the 4th and 9th passage.

^{45}Ca-washout Experiments were carried in six-well tissue culture boxes at 37°C. Before using the cells for experiments they were washed with physiological salt solution (PSS) three times and preincubated in this solution for at least 30 min. After preincubation the solution was removed by suction and the cells were incubated in PSS containing $^{45}CaCl_2$ (8 µCi/ml, Amersham) for 30 sec. The solution was removed and the cells were incubated for 60 sec in PSS and high-K solutions containing the same amount of $^{45}CaCl$ as before. The solutions were then removed and the cells were washed with ice-cold La^{3+}-wash solution twice every 30 sec for different time periods as indicated in the results. Subsequently, the cells were trypsinized for 5-10 min in 0.1% trypsin solution. A sample of each well was taken for determination of the cell number by Coulter counter. The rest of the trypsinated cells were transferred to scintillation vials and the ^{45}Ca-content was counted in a liquid spectrophotometer. 50 µl samples of each incubation solution was taken to determine the ^{45}Ca-content in the incubation solution. The cellular content of labeled Ca^{2+} is expressed as pmoles Ca^{2+} / 10^5 cells by deviding the c.p.m. in the cells by the specific radioactivity in the solution.

^{45}Ca-uptake Prewashed cells were incubated in PSS, high K solution or in PSS containing angiotensin II (10^{-6} M) labeled with ^{45}Ca for different time periods. The cells were then washed six times (90 sec) with ice cold La^{3+}-wash solution and analyzed as described above.

^{45}Ca-influx After preincubation the cells were first exposed for 30 sec to ^{45}Ca labelled PSS followed by a one minute exposure to solutions containing high K^+, 10^{-6} M angiotensin II or 10^{-6} M vasopressin all labeled to the same radiospecificity. The controls were labeled in PSS for 90 sec. The cells were then washed and counted as described above. For testing the effects of voltage gated channel blockade $5x10^{-8}$ M PY 108-068 was added 30 sec before and during the application of ^{45}Ca.

Solutions Physiological salt solution (PSS): 140 mM NaCl, 4.5 mM KCl, 1.0 mM $MgCl_2$, 1.5 mM $CaCl_2$, 1.8 mM glucose and 5 mM Hepes. High K-solution: 130 mM KCl, 14.5 mM NaCl, other components as in PSS. La^{3+}-wash solution: 5 mM $LaCl_3$,

0 mM $CaCl_2$, other components as in PSS. Trypsin solution: Ca-free Dulbeccos phosphate buffered saline containing 0.1% trypsin and 1 mM EDTA.

Drugs PY 108-068 (Sandoz AG); angiotensin II and vasopressin were purchased from Sigma and trypsin from Fakola AG, Basel.

RESULTS Figure 1 shows that frequent washing with a cold La^{3+}-solution for a short time period removes sufficient extracellular $^{45}Ca^{2+}$ to reveal stimulation of cellular $^{45}Ca^{2+}$-uptake by high K^+-depolarization. These short washout curves are composed of two exponential components with rate constants of 8.3 min^{-1} and 0.13 min^{-1}. For the interpretation of the subsequent experiments we assumed that the very rapid component (t 1/2 < 5 sec) represents ^{45}Ca adhering to the culture dish and cell surfaces and that the cellular ^{45}Ca is included in the slower component.

Figure 2 shows the time course of ^{45}Ca-uptake in control PSS and during maintained stimulation by high K^+ and angiotensin II. Compartmental analysis of the control curve revealed that, after an initial uptake of 20% of the

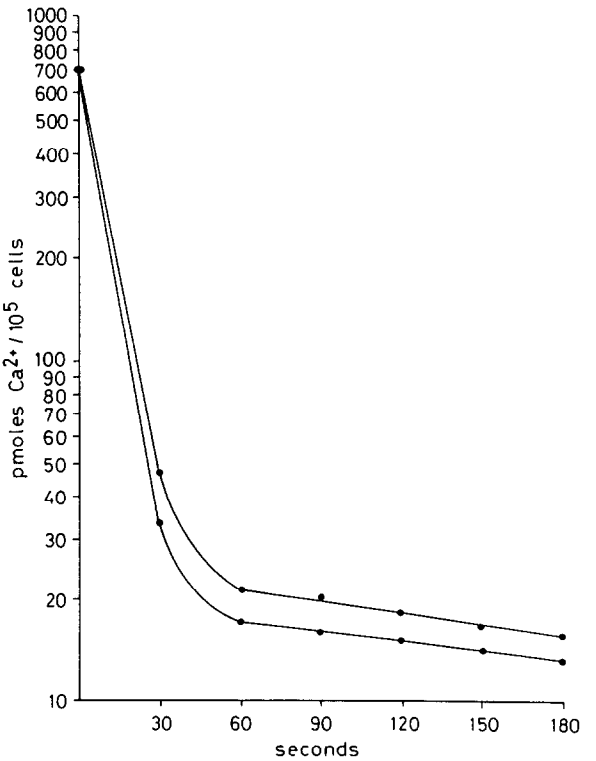

Figure 1: Washout of ^{45}Ca labeled Ca^{2+} from cultured smooth muscle cells loaded for 90 sec in high K^+ PSS (upper curve) or PSS (lower curve). Each point is the average of 4 determinations. The S.E. of the means are less than 10%.

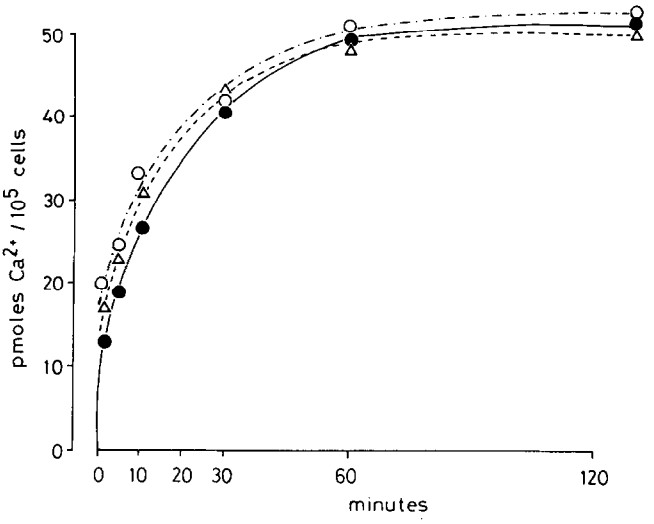

Figure 2: Time courses of $^{45}Ca^{2+}$-uptake, in control PSS (●), in high K^+ solution (Δ) or in the presence of angiotensin II (○). Each point is the average of 4 determinations. The S.E. of the means are 10% or less.

exchangeable Ca^{2+} at a rate too fast to be measured, the ^{45}Ca-uptake proceded with a single rate constant of 0.05 min^{-1}.

The effects of high K^+-depolarization and angiotensin II were to increase the rate of Ca^{2+}-influx without a significant effect on the equilibrium value of ^{45}Ca-uptake. At room temperature (data not shown) these agents also caused a net increased in Ca^{2+}-content.

Since some excitable Ca^{2+}-channels inactivate rapidly (15) we deemed it necessary to equilibrate the extracellular space with ^{45}Ca before stimulation in order to measure the full stimulation of ^{45}Ca-influx (see Methods). The results depicted in Figure 3 show that high K^+, angiotensin II and vasopressin all significantly stimulate ^{45}Ca-influx. The high K^+, but not the angiotensin II and vasopressin stimulated influx is blocked by $5x10^{-8}$ M PY 108-068. In addition it can be seen that the high K^+-stimulated ^{45}Ca-influx is additive to that stimulated by angiotensin II. In contrast, vasopressin does not enhance the ^{45}Ca-influx after it has been stimulated by angiotensin II.

DISCUSSION Excitable Ca^{2+}-channels provide the critical link between external vasopressor stimuli and intracellular activation of actomyosin (1,2,9). This study shows that in monolayers of cultured vascular smooth muscle cells the ^{45}Ca-fluxes through these channels may be recorded under physiological conditions.

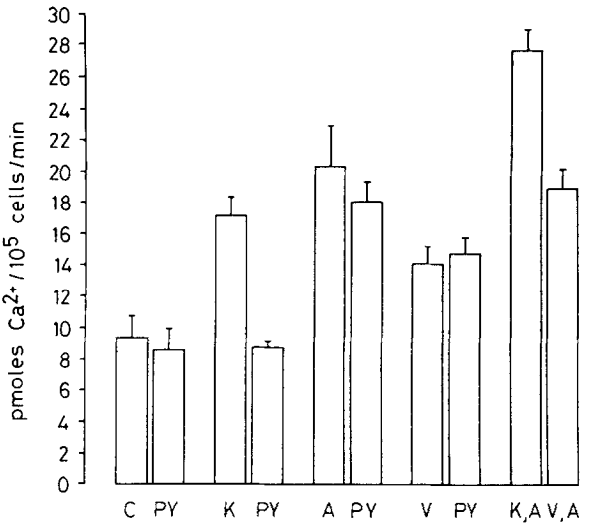

Figure 3: ^{45}Ca-uptakes by cultured smooth muscle cells during a 90 sec exposure to ^{45}Ca-labeled PSS (C) or during 30 sec to labeled PSS followed by 1 min labeled high K^+ PSS (K), 10^{-6} M angiotensin II (A), 10^{-6} M vasopressin (V), 10^{-6} M angiotensin in high K^+ PSS (K,A) or a combination of vasopressin and angiotensin (V,A). The columns labeled PY indicate addition of $5x10^{-8}$ M PY 108-068 to the solution indicated to the left. The bars signify S.E. of the means of at least 6 determinations.

The facts that the time course of ^{45}Ca-uptake after the first fraction of a minute could be fitted to a single exponential process and that stimulation did not affect the steady state ^{45}Ca-equilibration assures that differences in the initial ^{45}Ca-uptakes reflect stimulation of ^{45}Ca-influx. This point is furthermore substantiated by the complete blockade of the high K^+-stimulated ^{45}Ca-uptake by a known dihydropyridine Ca^{2+}-channel blocker PY 108-068. At the maximally effectve concentration for inhibition of voltage gated Ca^{2+}-channels, PY 108-068 had no effect on the ^{45}Ca-influx stimulated by either angiotensin II or vasopressin. In this respect the vasopressor activated Ca^{2+}-fluxes resemble the unitary cation currents activated by thrombin in platelets and endothelium (10,11), ATP in smooth muscle cells (9) and in neutrophils (8). These findings indicate that both angiotensin II and vasopressin activate receptor operated Ca^{2+}-channels.

Further evidence supporting the separate identity of vasopressor peptide activated channels and voltage gated Ca^{2+}channels is derived from the observation in figure 3 that at maximally effective doses of high K^+ and angiotensin II the stimulated ^{45}Ca-fluxes are additive. No such additivity is seen for the ^{45}Ca-fluxes stimulated by angiotensin II and vasopressin. A similar lack of additivity has been observed for ^{45}Ca-influx in the rabbit aorta stimulated by norepinephrine, histamine and serotonin (16).

These data suggest the interesting possibility that some types of receptors may be linked to the same population of receptor operated channels. The common link may be activation of the same G proteins and/or utilization of common second messengers. This group of receptor operated channels could thus be distinct from those such as the acetylcholine receptor (17), the GABA receptor (18) and the ATP activated Ca^{2+}-channel (9) where the agonists are directly involved in the gating of a channel associated with the receptor.

Even though the link between the vasopressor peptide receptors and receptor operated channels remains to be elucidated the present findings help to explain a current clinical problem. Ca-antagonists have already proven to be extremely valuable in the treatment of hypertension, especially that seen in the older low renin type patients (19). However, the younger high renin type of hypertensive patient has been found to be less responsive to Ca^{2+}-antagonists (20). The mechanism which underlies this latter clinical observation is most likely related to our finding that the angiotensin II stimulated Ca^{2+}-influx is resistant to dihydropyridines.

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