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VASOPRESSIN INCREASES $^{45}CA^{2+}$ INFLUX IN RAT AORTIC SMOOTH MUSCLE CELLS

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[Arg^8]Vasopressin (AVP)-induced $^{45}Ca^{2+}$ influx was examined in vascular smooth muscle cells derived from rat aorta. AVP stimulated the $^{45}Ca^{2+}$ influx in a concentration-dependent manner. The effect was abolished in the presence of La^{3+}. The dihydropyridine calcium channel antagonist darodipine did not affect the AVP-induced influx of $^{45}Ca^{2+}$. These data suggest that AVP stimulates in these cultured aortic smooth muscle cells a receptor-operated channel (ROC) that is permeable to Ca^{2+}.

An increase of cytoplasmic calcium in smooth muscle cells initiates activation of myosin ATP-ase and, therefore, contraction. Several contractile agonists have been shown to stimulate the formation of inositol (1,4,5)-trisphosphate (IP_3), which can discharge Ca^{2+} from an intracellular store, probably the sarcoplasmic reticulum (1-3). Extracellular Ca^{2+} can enter vascular smooth muscle cells by at least four different membrane transport mechanisms: a) Ca^{2+} leak systems, which are insensitive to organic Ca^{2+} antagonists, but can be inhibited by La^{3+} (4); b) voltage-sensitive Ca^{2+} channels of the so-called T (transient) and L (long-lasting) subtypes, the latter of which are sensitive to dihydropyridines in the nM concentration range (5-8); c) channels activated by agonists, so-called receptor-operated channels (ROC's; 9,10), which appear to allow the entry of both Ca^{2+} and Na^+ into the cells (11), and whose sensitivity to Ca^{2+} antagonists is debatable (11-13); and d) stretch-induced Ca^{2+} influx pathways, which are selectively inhibited by certain Ca^{2+} antagonists but not others (14).

[Arg^8]Vasopressin (AVP), a peptide originating from the hypothalamus, can cause contraction of vascular smooth muscle in vitro at concentrations as low as 10^{-12}M (15). AVP has been shown to stimulate the formation of IP_3 and to release Ca^{2+} from intracellular stores in vascular smooth muscle cells (16). This study investigates the effect of AVP on the entry of Ca^{2+} from extracellular sources into cultured vascular smooth muscle cells. In vascular smooth muscle, agonist-induced $^{45}Ca^{2+}$ influx has been studied in whole tissue preparations (4,12,13,17). Measurements in

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whole tissues, however, are complicated by the presence of extracellular matrix and of other cell types. The advantage of cultured cells is that a relatively homogenous population can be studied directly. For the first time, we can now provide evidence that aortic smooth muscle cell cultures can be used as a model for investigations of agonist-induced Ca^{2+} influx mechanisms.

Materials and Methods

Materials: $^{45}CaCl_2$ was obtained from Amersham International, England. [Arg^8]Vasopressin and angiotensin II were obtained from Bachem Ltd., Bubendorf, Switzerland. Darodipine (PY 108-068) was prepared by Sandoz Ltd., Basle. All other chemicals used were of the highest purity grade available.

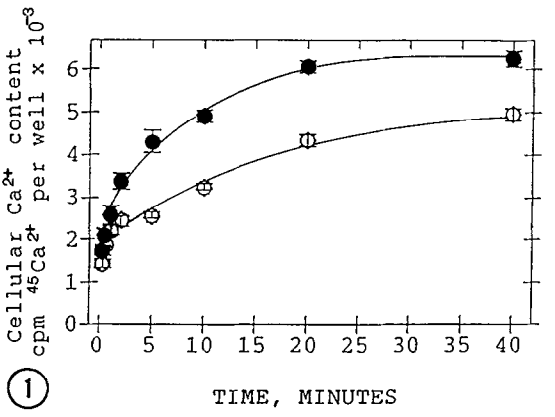
Cell culture: An aorta taken from a 12 week old Wistar-Kyoto rat was processed essentially as previously described (18). This method yields small numbers of cells but eliminates endothelial cells and fibroblasts. The cells were plated at a density of 3500 cells/cm^2 either in large surface bottles (for feeder cultures) or in 16 mm diameter wells (for flux experiments). They were grown in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum and kept at 37°C in a humidified atmosphere under 5% CO_2/95% O_2. Confluent feeder cultures were detached with 0.25% trypsin. For flux studies, cells were used 7 to 12 days after plating. In general, cell cultures with 2 to 10 passages yielded better signals than cells with more passages.

Ca^{2+}-influx measurements: Cells were preincubated for 10 min in 0.2 ml of Hepes buffered physiological salt solution (HBPSS, containing in mmol/l: NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1.2, Hepes 5, glucose 10, pH 7.4 at 37°C). In the experiments where darodipine was used, the Ca^{2+} antagonist was included during this 10 min period. $^{45}Ca^{2+}$ influx measurements were initiated by adding AVP or another agonist together with 1-3 μCi of $^{45}Ca^{2+}$ and the cells were incubated for time periods varying from 15 sec to 40 min (see figure legends for the specific time periods used in each protocol). After subsequent washing four times with ice-cold HBPSS containing no $CaCl_2$ but 0.2 mM EGTA (a maximum duration of 10 sec), the cells were solubilized with 0.25 ml of 0.1% sodium dodecylsulfate. The radioactivity of the lysate was measured by scintillation counting.

Statistical Evaluation: $^{45}Ca^{2+}$ uptake curves have been fitted using a least squares procedure to a single exponential function of the form $A_t = A_0 - A_0 e^{(-kt)}$, (where A_0= the capacity of the Ca^{2+} pool measured, k= its rate of uptake, and t= time) and compared between control and experimental conditions using analysis of variance. Mean values for $^{45}Ca^{2+}$ influx for individual measurements have been compared using Student's t-test for unpaired data. The concentration of AVP which was 50% effective (EC_{50}) was determined by estimation from its concentration response curve.

Results

Exposure of the cell cultures to [Arg^8]vasopressin led to an increase in the rate of Ca^{2+} uptake (k= 0.160 ± 0.029 min^{-1} as compared to k= 0.061 ± 0.019 min^{-1} in AVP-treated vs. control cells) but not to a significantly larger pool capacity (6152 ± 320 vs. 5268 ± 458 cpm/well in AVP-treated and control cells respectively). A significant increase in cellular $^{45}Ca^{2+}$ content between control and AVP-treated cells could be seen after two minutes. In the presence of the dihydropyridine Ca^{2+} antagonist darodipine (PY 108-068; 0.1μM), the kinetics of $^{45}Ca^{2+}$ uptake in both control and



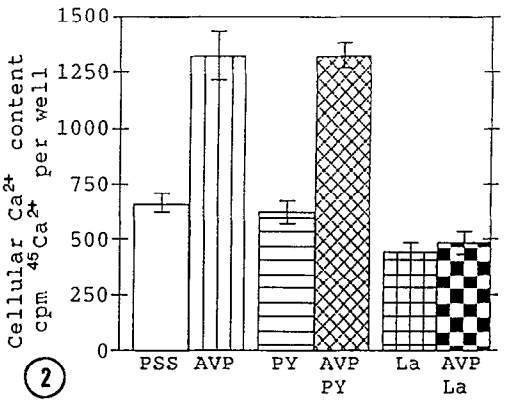


Figure 1. Time course of cellular $^{45}Ca^{2+}$ accumulation. Cells were incubated in HBPSS alone (-o-) or in this buffer containing AVP (0.1 μM) (-●-). As tracer, 3 μCi/ml of $^{45}Ca^{2+}$ was used. Results are expressed as mean ± S.E.M. cpm/well for n=8.

Figure 2. Effect of an organic (darodipine, PY 108-068 = PY; 0.1 μM) and an inorganic ($LaCl_3$, = La; 1mM) calcium entry blocker on control and AVP (0.1 μM)-induced $^{45}Ca^{2+}$ influx. Cellular $^{45}Ca^{2+}$ content was measured after 4 minutes and 1 μCi/ml of $^{45}Ca^{2+}$ was used as tracer. Results are expressed as mean ± S.E.M. cpm/well for n=4.

AVP-treated cells were unaffected (data not shown). This concentration of darodipine completely abolishes 55 mM K^+-stimulated $^{45}Ca^{2+}$ uptake in these or A_7r_5 cells (19). The inability of darodipine to decrease AVP-stimulated $^{45}Ca^{2+}$ influx is depicted in Figure 1; however, the inorganic channel blocker La^{3+} completely blocked AVP-stimulated $^{45}Ca^{2+}$ influx (Fig. 2).

The concentration-reponse curve for AVP-stimulated $^{45}Ca^{2+}$ influx (as measured during a stimulation time of 8 min) is shown in Figure 3. The EC_{50} is approximately 2×10^{-9}M.

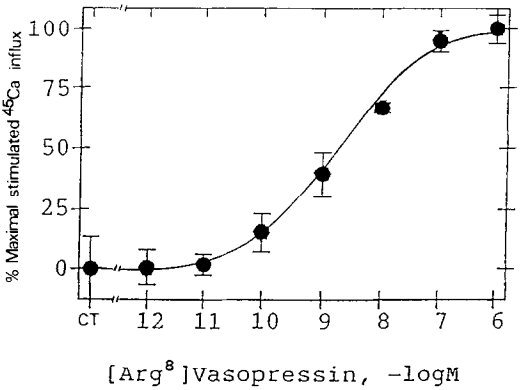


Figure 3. Dose response curve for the stimulation of $^{45}Ca^{2+}$ influx by AVP. Cells were incubated with AVP and $^{45}Ca^{2+}$ (1.2 μCi/ml) for 8 minutes. 0 % influx represents the value obtained in HBPSS; 100 % influx is the value for 1 μM AVP. Results are expressed as mean ± S.E.M. cpm/well for n=8.

Table 1. Effect of different agonists on $^{45}Ca^{2+}$ influx

Conditions		Cellular $^{45}Ca^{2+}$ content cpm per well	
		mean	S.E.M.
HBPSS		817	± 70
[Arg^8]vasopressin	1 μM	1339	± 19
Angiotensin II	1 μM	1127	± 78
Bradykinin	1 μM	1377	± 61
Serotonin	100 μM	954	± 24
Histamine	100 μM	761	± 38

Same experimental conditions as in Fig. 2; darodipine (0.1 μM) was present in all experiments. n=4.

The effects of two other peptides (angiotensin II and bradykinin) and two biogenic amine agonists (serotonin and histamine) on $^{45}Ca^{2+}$ influx stimulation were also determined (Table 1). The peptides were able to significantly stimulate the influx of $^{45}Ca^{2+}$ over control levels; the amines, however, did not significantly alter $^{45}Ca^{2+}$ influx. We were also unable to detect any increase in $^{45}Ca^{2+}$ influx with treatment of the cells with the α_1-adrenoceptor agonist phenylephrine (data not shown).

Discussion

Receptor-operated Ca^{2+} channels have been studied by several groups (9,10,17) in tissue preparations derived from smooth muscle. In isolated smooth muscle cells from rabbit ear artery, Benham & Tsien (11) have recently described an agonist-activated Ca^{2+}-current; underlying this current is the opening by the putative sympathetic nervous system cotransmitter ATP (20) of cation channels that are permeable to Ca^{2+}. These channels are not voltage-sensitive, and, unlike voltage-sensitive Ca^{2+} channels are not sensitive to inhibition by the dihydropyridine nifedipine. These channels appear to be the first clearly demonstrated single receptor-operated channels (ROC's) in vascular smooth muscle.

In this study we present $^{45}Ca^{2+}$ flux measurements which provide evidence for the existence of a receptor-operated Ca^{2+} influx pathway in cultured rat aortic smooth muscle cells. This $^{45}Ca^{2+}$ influx pathway is insensitive to dihydropyridine inhibition, as is the ATP-activated ROC, (11) but was blocked by La^{3+}. We suggest, therefore, that AVP and the other peptides presently studied open Ca^{2+}-permeable channels other than voltage-sensitive Ca^{2+} channels, and perhaps similar to the ATP-activated ROC. Nevertheless, the exact mechanism that allows Ca^{2+} to enter the cultured aortic cells in response to stimulation by such agonists as vasopressin, angiotensin II, and bradykinin cannot be deduced from ion tracer fluxes studies alone; other methods, such as the patch clamp electrophysiological technique, are required.

Because of the insensitivity of AVP-stimulated $^{45}Ca^{2+}$ influx to the dihydropyridines, we exclude an activation of voltage sensitive Ca^{2+} channels. An influx of Ca^{2+} due to activation of kinase C is unlikely, since kinase C-mediated $^{45}Ca^{2+}$ influx is also dihydropyridine sensitive (unpublished results). It is possible that a second messenger, such as IP_3 or inositol (1,3,4,5)-tetrakisphosphate (IP_4) is a mediator of Ca^{2+} entry in smooth muscle cells. Since IP_3 increases the Ca^{2+} permeability of the membrane of the sarcoplasmic reticulum (2), a similar effect is quite possible at the plasma membrane. Moreover, Irvine & Moor (21) suggest that IP_4 stimulates Ca^{2+} entry in sea urchin eggs. Perhaps a similar mechanim occurs in smooth muscle. On the other hand, Ca^{2+}entry may result from direct activation of the channels by the agonists, as found by Benham & Tsien for ATP (11). In such a mechanism, G-proteins could be involved (22).

The method presently used for studying the influx of $^{45}Ca^{2+}$ eliminates the complications of the presence of non-smooth muscle cell types and of significant quantities of connective tissue which are virtually unavoidable in whole tissues. This advantage simplifies to some extent the interpretation of $^{45}Ca^{2+}$ uptake data. The influence of vasopressin on the kinetics of $^{45}Ca^{2+}$ uptake (Fig. 1) in the cultured cells is similar to that of norepinephrine in rat aortic rings and mesenteric resistance vessels (17,23), wherein the agonist increased the rate of $^{45}Ca^{2+}$ uptake but not the steady-state content of $^{45}Ca^{2+}$. It is notable that the dihydropyridine darodipine has no effect on norepinephrine-induced contraction (24) or $^{45}Ca^{2+}$ influx (25) in the rabbit aorta, as it had no effect on AVP-stimulated influx in the cultured cells.

The absence of an effect of phenylephrine, serotonin and histamine on $^{45}Ca^{2+}$ influx in the cultured aortic cells parallels their weak effect on stimulation of $^{45}Ca^{2+}$ efflux in the cultured cells (16 and unpublished data), a stimulation being indicative of release of intracellular $^{45}Ca^{2+}$. The effect of these agonists on $^{45}Ca^{2+}$ influx and efflux are, however, readily detected in whole rabbit aorta (26). These observations suggest either that the receptors for these amines are lost during cell culture, or that the coupling between receptor-occupation and effector systems are altered for the amine receptors. The peptide receptors, in contrast, appear to retain their function in a manner similar to that expected in whole tissues in the stimulation of both Ca^{2+} influx and release of intracellular $^{45}Ca^{2+}$ (26,27).

In conclusion, we present here the first evidence that cultured rat aortic smooth muscle cells can be used as a model for investigations of agonist-induced Ca^{2+} influx mechanisms.

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