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Evidence for Two Separate Ca^{2+} Pathways in Smooth Muscle Plasmalemma

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Summary. The activation of rabbit aortic smooth muscle was studied by two most widely used vascular smooth muscle stimulants: α-adrenoceptor activation by norepinephrine (NE) and high-K^+ depolarization. This was studied by measurements of isometric contractions and net as well as unidirectional Ca^{2+} fluxes. These parameters showed markedly differential sensitivities towards two smooth muscle inhibitors used in this study: D600 and amrinone. By choosing an appropriate concentration of D600 or amrinone, Ca^{2+} uptake or Ca^{2+} influx induced by high K^+ or NE could be selectively inhibited. Furthermore, by using unidirectional flux measurements it was demonstrated that Ca^{2+} influx stimulated by NE and high K^+ were additive in nature. The data from the addivity experiment exclude the interpretation of a common Ca^{2+} pathway with two separate mechanisms for opening it. The data on three criteria employed in this study provide evidence for the existence of two independent Ca^{2+} pathways, one for each mode of activation, for Ca^{2+} influx known to be associated with these contractions.

The role of Ca^{2+} as an essential link in muscle contraction (skeletal, cardiac, and smooth muscle) is well established. Furthermore, Ca^{2+} is needed in various other physiological phenomena such as neurotransmitter release and exocrine gland secretion. Thus, the next step in improving our understanding of excitation-function coupling in general and excitation-contraction coupling in smooth muscle in particular is to investigate the characteristics of Ca^{2+} mobilization processes stimulated by different modes of activation.

Norepinephrine (NE) and high-K^+ depolarization represent the two most prominent means of activation of vascular smooth muscle; the former acting by binding to its specific α-adrenergic receptors on the smooth muscle membrane and the latter by depolarization of the cell membrane which is not related to any specific receptor activation. Thus, NE and high-K^+ induced contractions have been extensively utilized to study the details of the role of Ca^{2+} in the activation of vascular smooth muscle. In several vascular smooth muscle preparations, it has been observed that the removal of extracellular Ca^{2+} inhibits high-K^+ contractions to a greater extent than NE contraction (Waugh, 1962; Hinke, 1965; Hudgins & Weiss, 1968). This was demonstrated conclusively in a later study by the use of lanthanum (La^{3+}) to block transmembrane Ca^{2+} movements; it was observed that there was a distinct differential effect of La^{3+} on NE and high-K^+ concentrations (van Breemen, 1969). Similarly, a selective blockade of high-K^+ contractions of rabbit aorta by an organic Ca^{2+} antagonist, SKF 525A, was also demonstrated (Kalsner, Nickerson & Boyd, 1970).

A concept has emerged from the studies utilizing measurements of Ca^{2+} fluxes in vascular smooth muscle that both NE and high-K^+ depolarization induced contractions utilize influx of Ca^{2+} from the extracellular space, and that NE induced contractions utilize intracellular Ca^{2+} in addition to the Ca^{2+} influx (Deth & van Breemen, 1974, 1977; Bose & Innes, 1975). It has been suggested that Ca^{2+} influx during each of these two stimulations occurs through separate pathways (for review, *see* Bolton, 1979; van Breemen, Aaronson & Loutzenhiser 1979). However, the evidence available at present in support of this hypothesis is, at best, circumstantial. We now present evidence by direct measurements of Ca^{2+} fluxes indicating that there are two independent pathways through which Ca^{2+} can move across the cell membrane, one coupled to high-K^+ membrane depolarization and the other associated with the activation of α-adrenoceptors.

Three criteria were employed in the present study to demonstrate that NE and high-K^+ depolarization

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use separate pathways to mediate Ca^{2+} influx during the activation of vascular smooth muscle: (i) The use of inhibitors, D600 and amrinone, which demonstrate differential effects on contractions *as well as* cellular Ca^{2+} uptake. D600 is a well-known Ca^{2+} antagonist in smooth muscle (Fleckenstein, 1977; van Breemen & Siegel, 1980). We have recently shown that amrinone, a positive inotropic agent in heart, causes nonspecific inhibition of smooth muscle contractility (Meisheri, Palmer & van Breemen, 1980*a*). (ii) Measurements of unidirectional Ca^{2+} fluxes to demonstrate selective inhibition by D600 of the increase in Ca^{2+} influx induced by NE and high-K^+. (iii) Measurements of unidirectional Ca^{2+} fluxes to show that the Ca^{2+} influx stimulated by NE and high K^+ are additive in nature.

A preliminary report of these findings has been published (Meisheri, Hwang & van Breemen 1980*b*).

Materials and Methods

General

Adult rabbits were killed by a blow to the neck, their aortas rapidly excised and transferred to a physiological salt solution (PSS), bubbled with 100% O_2, and kept at 37 °C. The tissue was cleaned and cut into rings of 3–5 mm width.

Contractions

Isometric tension was measured by suspending the preparation between two stainless steel hooks, one of which was attached to the end of an aerator and the other connected to a force transducer which, in turn, was connected to a Grass Polygraph. Solutions were changed by switching the beakers in which the aortic rings were suspended. The inhibitory effects of D600 and amrinone were studied by first pretreating the tissue (10 min for D600 and 5 min for amrinone) and then exposing the tissue to NE or high-K^+ which also contained the appropriate inhibitor. The pretreatment schedules chosen were based on our previous studies (Meisheri et al., 1980*a*; van Breemen & Siegel, 1980).

Ca^{2+} Uptake

Parallel experiments were carried out to study the effects of D600 or amrinone on net ^{45}Ca uptake induced by the contractile agents. The muscle rings were exposed to ^{45}Ca labeled PSS for 90 min in order to label all the exchangeable cellular Ca^{2+}. This was followed by exposure to either control or an experimental solution also labeled with ^{45}Ca at the same specific activity. To be consistent with the contraction experiments, tissues were pretreated with the inhibitory agent (10 min for D600 and 5 min for amrinone) before exposing them to either NE (10^{-5} M) or high-K^+ solution also containing the inhibitory agent. At the end of a 10-min exposure to the stimulating agent, the tissues were bathed in ice-cold Ca^{2+} free PSS containing 2 mM EGTA for 45 min in order to remove extracellular ^{45}Ca. The validity of this method has been discussed previously (Meisheri et al, 1980*a*). After this, the tissues were blotted, weighed, and incubated overnight in 3 ml of 5 mM EDTA at room temperature (*see* Meisheri et al., 1980*a*). Finally, 7 ml of scintillation cocktail containing Triton X-100 were added and vials analyzed for ^{45}Ca in a liquid scintillation counter. The data was expressed as Ca^{2+} uptake μmol/kg aorta (wet wt).

Ca^{2+} Influx

Measurements of unidirectional fluxes were also carried out to study NE and high-K^+ induced Ca^{2+} influx. For this, the tissues were exposed to ^{45}Ca containing solutions for short periods of time (3–10 min). The amount of ^{45}Ca entering the tissue during such short periods can be assumed to be primarily due to Ca^{2+} influx. This experimental approach allowed us to selectively determine the influence of NE or high-K^+ on the influx of Ca^{2+} from the extracellular space. Briefly, the aortic rings were incubated in nonradioactive PSS for up to 60 min. The rings were then exposed to control or experimental solutions containing ^{45}Ca, and tissues were taken out at different time periods up to 5 or 10 min as indicated in the Results section. The amount of ^{45}Ca entering the tissue was then determined as described in the previous section. This experimental protocol was designed to test the criteria of selective inhibition by D600 of Ca^{2+} influx and that of additivity of NE and high-K^+ induced Ca^{2+} influx.

Solutions

The solutions used were PSS (in mM): NaCl, 140; $CaCl_2$, 1.5; $MgCl_2$, 1; KCl, 4.6, glucose, 10; Hepes (N-2-hydroxy-ethylpiperazine-N′-2-ethanesulfonic acid) buffer, 5. The pH was adjusted to 7.2 by adding 1.0 N NaOH. High-K^+ depolarizing medium contained 80 mM KCl and 64.6 mM NaCl; other ingredients remained the same as above. Ca^{2+}-free PSS containing EGTA had 2 mM EGTA and $CaCl_2$ was deleted. A stock solution of amrinome (mol wt 187.2) was prepared in distilled water with the aid of conc. HCl. From this stock solution, desired concentration of amrinone was prepared in PSS, and pH was maintained at 7.2. Stock solutions of D600 and NE were prepared and added in appropriate volume into the muscle bath to give desired concentration.

Drugs and Chemicals

L-norepinephrine hydrochloride (Sigma), D600 (A.G. Knoll Pharmaceutical Laboratories), amrinone (Sterling Winthrop) ^{45}Ca (sp act 12.9 mCi/mg) was obtained from New England Nuclear.

Results

Dose-response curves depicting D600 and amrinone-induced inhibition of NE and high-K^+ contractions in rabbit aorta are shown in Fig. 1. Both D600 and amrinone caused a dose-dependent inhibition of contractions induced by NE or high-K^+. 80 mM K^+-induced contractions were significantly more sensitive than NE (10^{-5} M) to the inhibitory effect of D600, as is apparent from Fig. 1*A*. In contrast, the inhibition curves for NE (10^{-6} M) and 80 mM K^+ were similar in the presence of amrinone (Fig. 1*B*). The concentration range of amrinone used is similar to the concentration range in which amrinone is reported to produce a positive inotropic effect in cardiac preparations (Farah & Alousi, 1978). In Fig. 1*B*, the NE concentration used was 10^{-6} M. In preliminary experiments, when 10^{-5} M NE was used the % inhibition of contractions at 10 and 100 μg/ml of amrinone were similar to those obtained with 10^{-6} M NE.

Parallel experiments were carried out to study the effects of D600 or amrinone on net Ca^{2+} uptake induced by the contractile agents. The experiments

were designed such that the effects of an inhibitor (D600 or amrinone) on Ca^{2+} uptake induced by NE and high-K^+ could be studied on aortic rings from the same preparation. This allowed us to compare the inhibitory effects of D600 or amrinone on the Ca^{2+} uptake stimulated by the two stimulating agents simultaneously. Thus, it became necessary to generate the data from a series of experiments performed by using one or two concentrations of D600 or amrinone in each experiment. This also required the generation of control data points (i.e., net Ca^{2+} uptakes stimulated by 80 mM K^+ and NE, 10^{-5} M in the absence of the inhibitors) for each experiment. In order to normalize the data, the following procedure was used so that the dose dependency of the inhibitory effects of D600 or amrinone could be analyzed: The net Ca^{2+} uptake was measured as described in Methods, and the data were first calculated as Ca^{2+} uptake μmol/kg aorta net weight (*see* Tables 1 and 2). From these, average values for the increase in Ca^{2+} uptake over control by the stimulating agent, in the absence as well as presence of D600 or amrinone, were calculated. Percent inhibitions of Ca^{2+} uptake by the inhibitors were then calculated to generate Fig. 2*A* and *B*. Because of this method of computation, standard errors are not shown in the figures. Two tables giving raw data from representative experiments (one for D600 and one for amrinone) are included (*see* Tables 1 and 2). Note that, although the absolute values of net Ca^{2+} uptake by NE and high-K^+ vary from experiment to experiment, the average increases in Ca^{2+} uptake over control are comparable. As shown in Fig. 2*A*, the D600 dose-response curve for NE (10^{-5} M) activated Ca^{2+} uptake lies well to the right of the one for high-K^+ activated Ca^{2+} uptake. The IC_{50} of D600 (concentration of D600 producing 50% inhibition) for high-K $= 1.5 \times 10^{-7}$ M, whereas IC_{50} of D600 for NE $= 10^{-5}$ M. Comparison of Figs. 1*A* and 2*A* reveals that D600 was a more effective inhibitor of NE induced Ca^{2+} uptake than of NE induced

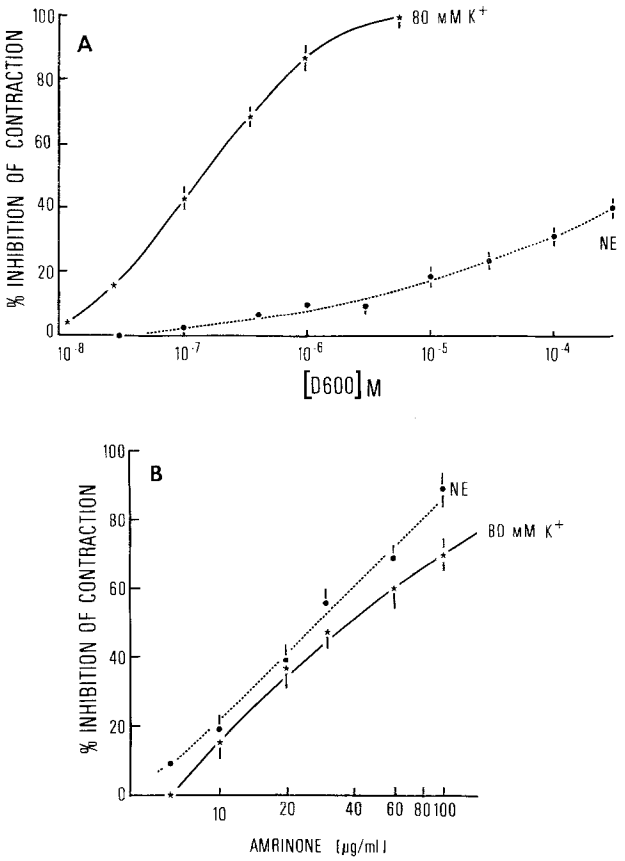

Fig. 1. The effects of D600 (*A*) and amrinone (*B*) on NE (10^{-5} M) or high-K^+ (80 mM) induced contractions of rabbit aorta. Isometric contractions were measured and recorded on a polygraph. The pretreatment period was 10 min for D600 and 5 min for amrinone. These pretreatment schedules were observed to produce the maximum inhibitory effect. The data for each point is given as mean ± SEM ($n=4$). *B* is taken from Meisheri et al. 1980*a*

contraction. The difference in the NE and high-K^+ induced Ca^{2+} uptake processes is exemplified further by the use of amrinone (Fig. 2*B*). Here the sensitivity is reversed. There was a very small effect of amrinone on high-K^+ induced Ca^{2+} uptake, the maximum inhibition observed being 28%. In contrast, the inhibition

Table 1. The effect of D600 (10^{-6} M) on NE (10^{-5} M) and high-K^+ (80 mM) induced net Ca^{2+} uptake in rabbit aorta[a]

Treatment	Net Ca^{2+} uptake (μmol/kg aorta)[b]	Increase over control (μmol/kg aorta)[c]	% Inhibition by D600
Control	195.0 ± 21.8 (6)		
80 mM K^+	297.0 ± 15.0 (6)	102.0 ± 26.5	
NE, 10^{-5} M	275.0 ± 15.6 (6)	80.0 ± 26.8	
D600 (10^{-6} M) control	180.0 ± 9.8 (6)		
D600 + 80 mM K^+	192.8 ± 7.2 (6)	12.3 ± 12.2	87.9
D600 + NE, 10^{-5} M	252.0 ± 15.0 (6)	72.0 ± 17.9	10

[a] Similar data were generated using a series of D600 concentrations and used to generate Fig. 2*A*
[b] The data given as mean ± SEM (n)
[c] The data given as mean ± SEM

Table 2. The effect of amrinone (80 μg/ml) on NE (10^{-5} M) and high-K^+ (80 mM) induced net Ca^{2+} uptake in rabbit aorta[a]

Treatment	Net Ca^{2+} uptake (μmol/kg aorta)[a]	Increase over control (μmol/kg aorta)[b]	%Inhibition by amrinone
Control	101.2 ± 5.5 (4)		
80 mM K^+	236.2 ± 9.7 (4)	135.0 ± 9.8	
NE, 10^{-5} M	176.7 ± 11.4 (5)	75.5 ± 11.5	
Amrinone (80 μg/ml) control	124.3 ± 7.0 (4)		
Amrinone + 80 mM K^+	229.2 ± 6.8 (5)	104.9 ± 9.8	22.3
Amrinone + NE, 10^{-5} M	147.0 ± 5.8 (4)	23.0 ± 9.1	69.5

[a] Similar data were generated using a series of amrinone concentrations and used to generate Fig. 2*B*
[b] The data given as mean ± SEM (*n*)
[c] The data given mean ± SEM

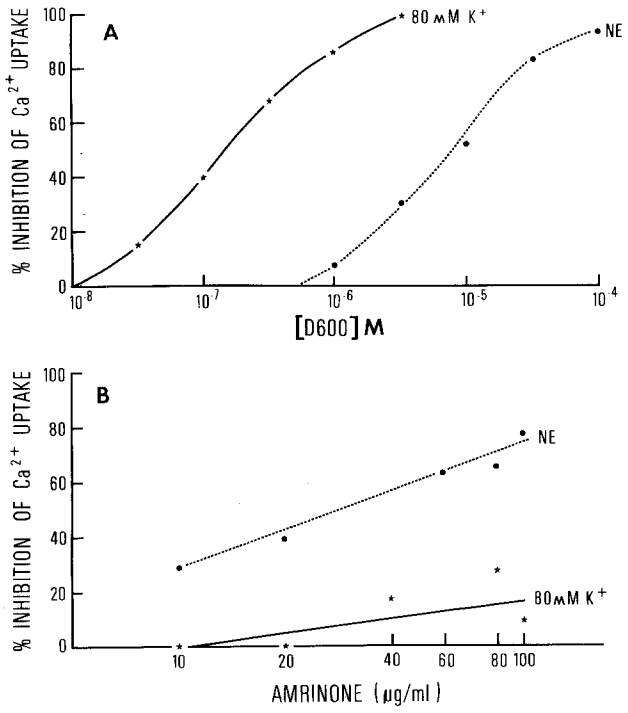

Fig. 2. The effect of D600 (*A*) and amrinone (*B*) on NE (10^{-5} M) or high-K^+ (80 mM) induced net Ca^{2+} uptake in rabbit aorta. The raw data from a representative experiment is given in the Tables. From these, the percent inhibition of Ca^{2+} uptake by the antagonists were calculated (as described in the Results) to generate this figure

of 10^{-5} M NE activated uptake by amrinone was significantly greater, ranging from 28 to 77%.

In order to demonstrate selective inhibition of Ca^{2+} influx by D600, unidirectional Ca^{2+} fluxes were measured as described in the Methods and the results are shown in Fig. 3*A*. A dose of D600 (10^{-6} M) was chosen from Fig. 2*A* in which 10^{-6} M D600 was shown to have a significant effect on high-K^+ induced Ca^{2+} uptake but very little influence on NE-induced Ca^{2+} uptake. Measurement of Ca^{2+} influx (Fig. 3*A*) clearly demonstrated that pretreatment with 10^{-6} M D600 caused a complete inhibition of high-K^+ induced Ca^{2+} influx by 5 min, whereas Ca^{2+} influx induced by NE remained unaffected.

The final test of our postulate comes from the criterion of additivity. For this unidirectional Ca^{2+} flux measurements were carried out again as described earlier. As shown in Fig. 3*B*, each of the two stimulating agents, NE (10^{-5} M) and high-K^+, caused a time-dependent increase in Ca^{2+} influx over control as expected. When both agents were added together, the stimulated Ca^{2+} influx was additive. In other words, at each time point the stimulated Ca^{2+} influx value over control obtained with NE and high-K^+ together approximated the value obtained by addition of stimulated Ca^{2+} influx over control by each agent alone.

The last two figures give the results of two control experiments. The criterion of additivity requires that at least one of the two treatments should produce the maximum effect on the response being studied. Figure 4 gives the results of an experiment designed to test this requirement. Unidirectional Ca^{2+} influx over a period of initial 5 min was determined with 10^{-5} M, 10^{-4} NE, 80 mM K^+ (+60 mM Na^+), and 145 mM K^+ (0 Na^+). The solution containing 145 mM K^+ represents maximal high-K^+ concentration, attainable without altering osmolarity or tonicity of the solution. Both high-K^+ solutions contained phentolamine (10^{-5} M) to avoid interference due to possible release of NE from the nerve terminal. As seen in Fig. 4, 10^{-5} M NE produced the maximum increase in Ca^{2+} influx, and no further increase in Ca^{2+} influx was observed at 10^{-4} M NE. Also, Ca^{2+} influxes stimulated by 80 mM K^+ and 145 mM K^+ depolarizing solutions were similar.

The other control experiment dealt with the fact that in experiments with high-K^+, a lowered Na^+ concentration was used with respect to control. A possibility remained that there may be a Na-dependent component of Ca^{2+}-influx which would complicate the interpretation of the data obtained with solutions containing lowered Na^+. The results shown in

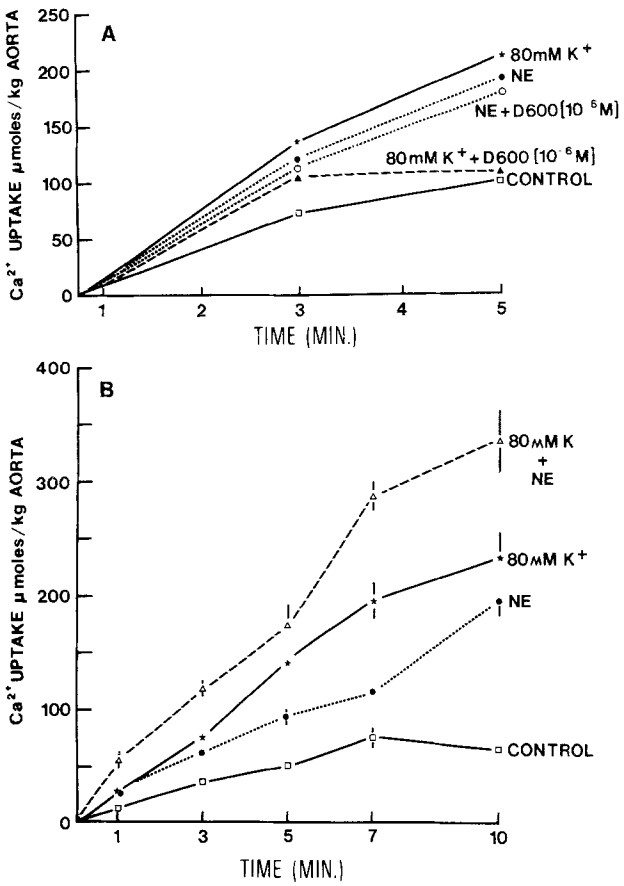

Fig. 3. (*A*): The effect of 10^{-6} M D600 on Ca^{2+} influx stimulated by NE (10^{-5} M) and 80 mM K^+ in rabbit aorta. Eight or nine aortic rings were used for each time point. The SEMs were 10 to 15% and have not been shown for sake of clarity. D600 caused a significant ($P \leqq 0.05$) inhibition of high-K^+ induced Ca^{2+} influx at both time points studied. (*B*): Ca^{2+} influx stimulated by NE (10^{-5} M), 80 mM K^+, and NE and high-K^+ together in rabbit aorta. The procedure for Ca^{2+} influx measurement is described in Materials and Methods. The results are shown as mean ± SEM ($n=4$ or 5). Although the absolute values of Ca^{2+} uptake appear different in *A* and *B*, the stimulated Ca^{2+} influx over control by each stimulating agent is comparable

Fig. 5 exclude such a possibility. Again, unidirectional Ca^{2+} influx was measured after a 5-min exposure to ^{45}Ca containing control or an experimental solution. When the external Na^+ was lowered (from 140 mM as in normal solution to 60 mM) neither control Ca^{2+} influx (columns 1 and 3) nor NE, 10^{-5} M, induced Ca^{2+}-influx (columns 2 and 4) was significantly altered. Similarly, Ca^{2+} influxes obtained with 80 mM K^+ in the presence of 60 mM Na^+ or 0 Na^+ (sucrose replacement) were not significantly different (columns 5 and 6). Thus, high-K^+-induced Ca^{2+} influx does not involve any Na-dependent Ca^{2+}-influx component under the above experimental conditions. The above two experiments (Figs. 4 and 5) confirm that the additivity of Ca^{2+} influxes observed in Fig. 3*B* does not represent any experimental artifacts.

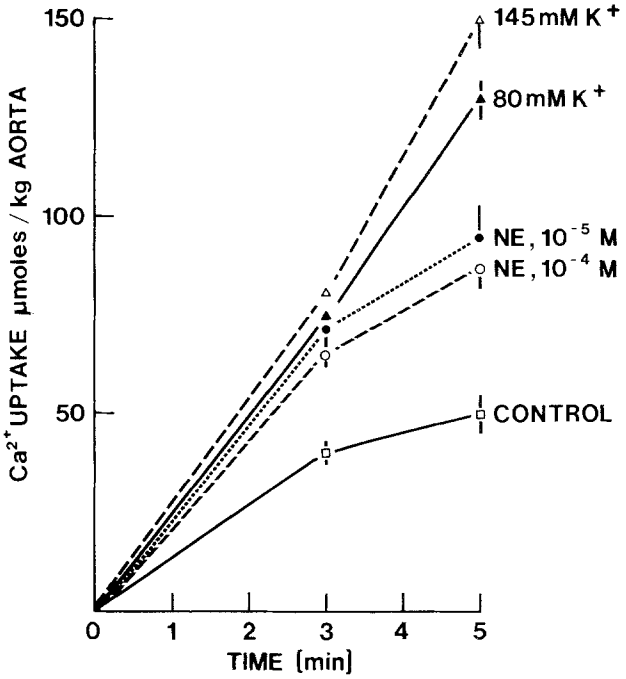

Fig. 4. Ca^{2+} influx stimulated by NE (10^{-5} and 10^{-4} M), 80 mM, and 145 mM K^+ in rabbit aorta. 80 mM K^+ solution contained 60 mM Na^+, whereas 145 mM K^+ contained no Na^+. Both high-K^+ solutions contained 10^{-5} M phentolamine. Each data point is given as mean ± SEM ($n=4$ or 5). (*See* text for details)

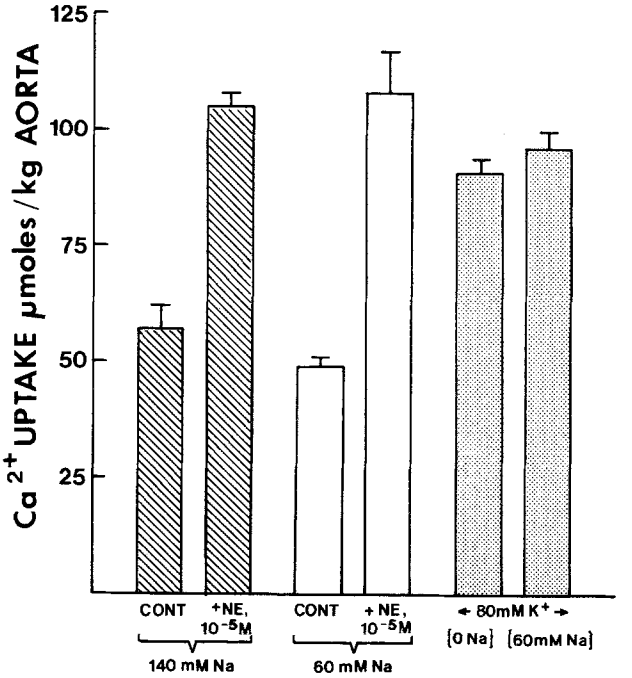

Fig. 5. Ca^{2+} influx stimulated by NE (10^{-5} M) and 80 mM K^+ in various solutions. All tissues were exposed to ^{45}Ca for only 5 min. The first two columns indicate a 5-min Ca^{2+} influx in normal PSS (140 mM Na^+): control and with 10^{-5} M NE. Columns 3 and 4 show the effect of lowered Na^+ (60 mM; and 148.8 mM sucrose to maintain isomolarity). The last two columns show Ca^{2+} influx stimulated by 80 mM K^+ (0 Na^+, 148.8 mM sucrose, and 10^{-5} M phentolamine) and 80 mM K^+ (60 mM Na^+ and 10^{-5} M phentolamine), respectively. Each bar graph is given as mean ± SEM ($n=5$). (*See* text for details)

Discussion

Three lines of experimental evidence provided in this study indicate that α-adrenoceptor stimulation induced by Ca^{2+} influx in vascular smooth muscle cell occurs via a different pathway than the Ca^{2+} influx induced by depolarization of the cell membrane with elevation of extracellular K^+ concentration. The first line of evidence consisted of demonstration of differential effects of D 600 and amrinone on Ca^{2+} mobilization processes stimulated by the two activation stimuli. Selective antagonists have been classical tools to demonstrate existence of ionic channels in cell membrane. For example, tetrodotoxin and tetraethylammonium ions have been used to selectively block and thus characterize Na^+ and K^+ channels (Ritchie, 1975; Hille, 1973). In the present study, although D 600 and amrinone did not appear completely selective for NE or high-K^+ activation, significant differences in sensitivities of these two stimuli towards inhibition by D 600 or amrinone were quite apparent. Thus, by choosing an appropriate concentration of D 600 or amrinone, we could selectively inhibit Ca^{2+} uptake induced by high-K^+ or NE. Such clearcut distinction was not apparent with amrinone-induced inhibition of NE or high-K^+ contractions. It has been pointed out that the use of contraction experiments to draw conclusions regarding Ca^{2+} movements in smooth muscle without simultaneous measurements of Ca^{2+} fluxes may be inappropriate (van Breemen, 1975). The above observation provides further support for this conclusion. Amrinone was shown to cause inhibition of high-K^+-induced contractions without significantly affecting high-K^+-induced Ca^{2+} uptake (this study and Meisheri et al., 1980*a*). This was attributed to a stimulatory effect of amrinone on intracellular Ca^{2+} sequestration in addition to its effects on membrane Ca^{2+} flux (Meisheri et al., 1980*a*). With regard to D 600, it should be noted that D 600 was found to be a more effective inhibitor of NE-induced Ca^{2+} uptake than of NE-induced contraction (*compare* Figs. 1*A* and 2*A*). This difference can probably be explained by the existence of the NE-induced intracellular Ca^{2+} release, which is relatively insensitive to D 600.

Further confirmation of the concept of a separate Ca^{2+} pathways in vascular smooth muscle comes from the measurements of unidirectional Ca^{2+} fluxes. The uniqueness of this experimental approach lies in that we could selectively examine the Ca^{2+} influx processes without the complicating factors of back fluxes. This also excludes the complication of intracellular Ca^{2+} release which is known to be associated with the action of NE in this tissue (Deth & van Breemen, 1977). Thus, selective inhibition by D 600 of extracellular Ca^{2+} mobilization across the plasmalemma was evident by measurments of net as well as unidirectional Ca^{2+} fluxes. The criterion of selective antagonism is thus fulfilled.

It is, however, possible to create a model which consists of one Ca^{2+} pathway and two separate mechanisms for opening the pathway, each of which is differentially inhibited by the inhibitors used in this study. The concept of only one pathway would imply that when this pathway is fully opened by exposure to the maximum stimulating concentration of one agent, no additional Ca^{2+} entry would be apparent when exposed to the other stimulating agent simultaneously. Our experiments clearly indicate this not to be the case. The measurements of initial rates of Ca^{2+} influx during high-K^+ and NE-activation (Fig. 3*B*) demonstrated that the increases in Ca^{2+} permeability due to high-K^+ and NE were additive when the two procedures of activation were applied simultaneously. Thus, application of this important criterion of additivity rules out the above model and confirms the postulate of two different and independent Ca^{2+} pathways in smooth muscle plasmalemma.

The presence of separate Ca^{2+} pathways for two different stimuli may indicate a refined system for activation of smooth muscle where one mechansim of activation can exist independent of the other. In this regard, it would be of great interest to investigate if separate Ca^{2+} pathways also exist for other agonists (histamine, angiotensin II, prostaglandins) that require influx of Ca^{2+} for activation of smooth muscle. The criteria employed in this study should prove useful for such an investigation.

Although the data presented here provide strong evidence for two separate Ca^{2+} pathways in the smooth muscle plasmalemma, the nature of these pathways remains elusive. These could be either channels in the membrane or carriers that transport Ca ions across the membrane. A description of the exact nature of these "Ca^{2+} pathways" awaits further work.

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