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Native-type DHP-sensitive calcium channel currents are produced by cloned rat aortic smooth muscle and cardiac α_1 subunits expressed in *Xenopus laevis* oocytes and are regulated by α_2- and β-subunits

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Native tissue-like L-type voltage-dependent calcium channels (L-VDCC's) were expressed by in vitro transcribed cRNA injection of rat aorta or rabbit cardiac α_1 subunit into *Xenopus laevis* oocytes. Co-injection of VSM-α_1 with the cloned skeletal muscle β-subunit (SK-β) of the L-type VDCC significantly increased the expressed peak current amplitude without significant changes in kinetics. Similar results were obtained by co-injection of cardiac α_1 (DSHT-α_1) the cloned skeletal α_2-subunit (SK-α_2) or with SK-β. The oocytes co-expressing cRNA's retained L-type VDCC pharmacology.

Voltage-dependent calcium channel; Tissue-specific subunit; Co-expression; *Xenopus laevis* oocyte

1. INTRODUCTION

Vascular smooth muscle (VSM) is the primary therapeutic target of the class of compounds known as the calcium antagonists, which are used to treat hypertension and angina pectoris. The drugs include the 1,4-dihydropyridines (DHP's), phenylalkylamines and benzothiazepines, which selectively bind to L-type voltage-dependent calcium channels (VDCC's) and produce an inhibition of calcium influx [1]. The L-type VDCC's located in VSM possess the highest affinity of any tissue for the calcium antagonists and are responsible for the potent vasodilatory effects seen by these agents [2,3].

DHP-sensitive VDCC's have been characterized by biochemical and molecular biological techniques. The rabbit skeletal muscle VDCC exists as a pentameric macromolecule consisting of the α_1, α_2-δ, β and γ polypeptide subunits [4–6]. The α_1-subunit is a large hydrophobic polypeptide which is now recognized as both the channel-forming component as well as the specific calcium antagonist receptor [7]. Several groups, including this laboratory, have cloned full-length L-type α_1-subunits from various sources including rabbit skeletal [8,9], carp skeletal muscle [10], cardiac [11,12] and airway smooth [13] muscles. We have recently reported the cloning of a VSM α_1-DNA isolated from rat aorta (VSM-α_1) [14]. This aortic cDNA is homologous to the rabbit cardiac and airway smooth VDCC, but with specific cytoplasmic differences. It appears that these two channels may arise from alternative splicing of the same gene [14,15].

In the present study, we have functionally expressed the aortic and cardiac VDCC's by the injection of cRNA's synthesized from the rat aortic and rabbit cardiac α_1-cDNA's into *Xenopus* oocytes. In addition to the expressed VDCC activity, which we find to be sensitive to both calcium agonists and antagonists, the co-injection of cRNA's derived from the cloned rabbit skeletal muscle α_2 [9] and β-subunit [16] cDNA's stimulates the α_1-directed calcium current.

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2. MATERIALS AND METHODS

2.1. *In vitro transcription*

The full-length rat aorta α_1 cDNA (VSM-α_1) was inserted as a 8.3 kb *Not*I-*Sal*I fragment into the plasmid vector pBluescript KS$^+$ (Stratagene, Inc., La Jolla, CA) as previously described [14]. This construct includes the 6.8 kb coding region and does not include a poly (A)$^+$ tract. The cDNA template was linearized by *Sal*I and cRNA was synthesized by T3 RNA polymerase. Rabbit skeletal muscle β-subunit cDNA [16] was first cloned into a modified SP64(poly A) plasmid (Promega Inc.) in which the *Eco*RI site was replaced with a *Kpn*I site. A partial digestion of the SK-β cDNA with *Hind*III and *Xba*I followed by the ligation into the *Hind*III-*Xba*I site of the modified SP64(poly A) vector resulted in our cRNA template. The β-subunit template was then linearized by *Kpn*I and cRNA was synthesized by SP6 RNA polymerase. Previously published cardiac clones [12] were used to screen a λZAPII library (Stratagene) to isolate clones which completed the coding frame, and these clones were used to construct a full length cardiac-α_1. The cardiac α_1 cDNA (DSHT-α_1, 7.1 kb) and the skeletal α_2 cDNA (SK-α_2, 3.8 kb) [9] were inserted into the *Hind*III-*Xba*I and *Sal*I-*Sac*I sites of the modified SP64 (poly A) plasmid, respectively.

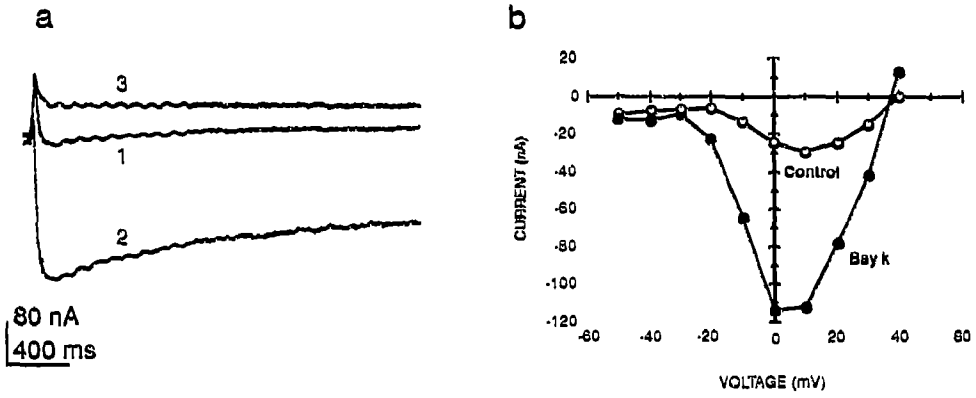

Fig. 1. I_{Ba} induced by VSM-α_1. (a) I_{Ba}, induced by VSM-α_1 (trace 1); enhancement by addition of 10^{-6} M Bay K 8644 (trace 2); inhibition by addition of 100 μM Cd^{2+} (trace 3). I_{Ba}'s were obtained by using a test pulse to 0 mV from a holding potential of −80 mV. (b) Current–voltage relationship of VSM-α_1 injected oocyte. The open and closed circles represent the peak current amplitudes at the indicated test potentials before and after Bay K 8644 treatment, respectively.

The corresponding cRNA's were prepared as described for SK-β. The cRNA's were purified by standard molecular biological procedures and suspended in water at a final concentration of 1 mg per ml.

2.2. *Oocyte injections and electrophysiology*

Adult female *Xenopus laevis* frogs were purchased from Xenopus One (Ann Arbor, MI) and were kept under light and dark cycles of 12 h. Injections of cRNA's were performed according to published procedures [17]. Briefly, oocytes were removed surgically and treated with type 1A collagenase (Sigma Chemical Inc.) in the following solution (in mM): NaCl, 82.5; KCl, 2.0; $MgCl_2$, 1.0; HEPES, 5.0 (titrated to pH 7.5) for 2 h at 20°C with gentle agitation. Oocytes at stage V–VI were selected and injected with 40 nl of the aqueous cRNA solutions. In oocytes that expressed two subunits, 80 nl of a 1:1 mixture of cRNA's were injected. Oocytes were incubated in a solution of the following composition (in mM): NaCl, 96.5; KCl, 2.0; $CaCl_2$, 1.8; $MgCl_2$, 1.0; HEPES, 5.0; pyruvate, 2.5; theophylline, 0.5; penicillin, 100 U/ml; streptomycin, 100 μg/ml (titrated to pH 7.4) at 20°C for 4 to 5 days before electrophysiological experiments were performed [18].

Calcium channel activity was detected and measured by the standard two-electrode voltage-clamp technique, usually 4 days after injection. Barium was substituted for calcium, external Cl^- was replaced by methanesulphonate, and whole cell currents were recorded at room temperature in the following solution (in mM); $Ba(OH)_2$, 40; *N*-methyl-D-glucamine, 50; KOH, 2.0; HEPES, 5.0, and the pH was adjusted to 7.4 with methanesulfonic acid. The electrodes were filled with 3 M KCl and had resistances of 1.0 – 3.0 MΩ. Cd^{2+}-insensitive currents were subtracted from the traces where they were mentioned. Data were filtered at 50 Hz and were analyzed by pCLAMP software package (Axon Instruments, Burlingame, CA). Diltiazem was a gift from Marion Merrell Dow, Bay K 8644 from Miles Laboratory and (±)PN200-110 from Sandoz.

3. RESULTS AND DISCUSSION

Ba^{2+} currents (I_{Ba}), expressed by VSM-α_1 cRNA alone, were enhanced by the addition of Bay K 8644 and blocked by Cd^{2+} (Fig. 1a). Non-injected and SK-β injected oocytes did not show any Bay K 8644-sensitive current. Fig. 1b shows the current–voltage relationship (I–V curve) of peak I_{Ba} of a VSM-α_1 injected oocyte. The maximum peak current was obtained at 10 mV and was shifted to 0 mV after the addition of Bay K 8644. I_{Ba}, expressed by VSM-α_1 cRNA co-injected with SK-β cRNA, was also enhanced by Bay K 8644 and was

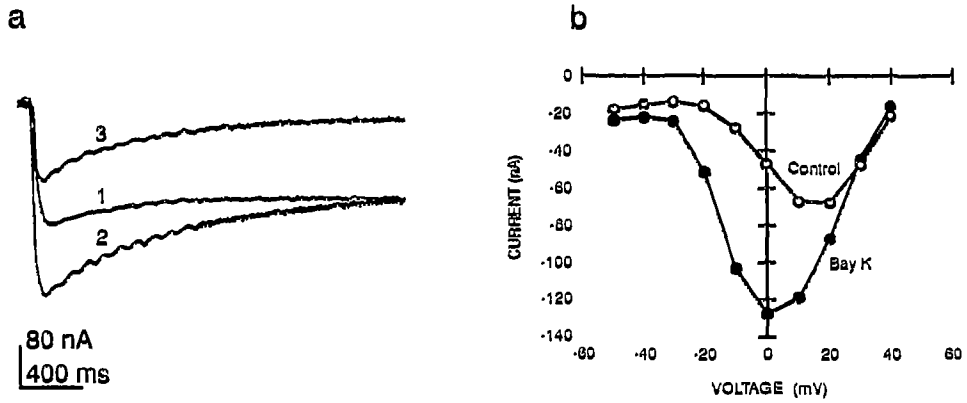

Fig. 2. I_{Ba} induced by co-injection of VSM-α_1 and SK-β. (a) I_{Ba}, induced by co-injection op VSM-91 and SK-β (trace 1). Enhancement by addition of 10^{-6} M Bay K 8644 (trace 2); suppression by 10^{-6} M diltiazem (trace 3). Cd^{2+}-insensitive currents were subtracted. The voltage-clamp protocol is the same as described in Fig. 1. (b) Current–voltage relationship of VSM-α_1 plus skeletal β co-injected oocyte. The open and closed circles represent the peak current amplitudes at the indicated test potentials before and after Bay K 8644 treatment, respectively.

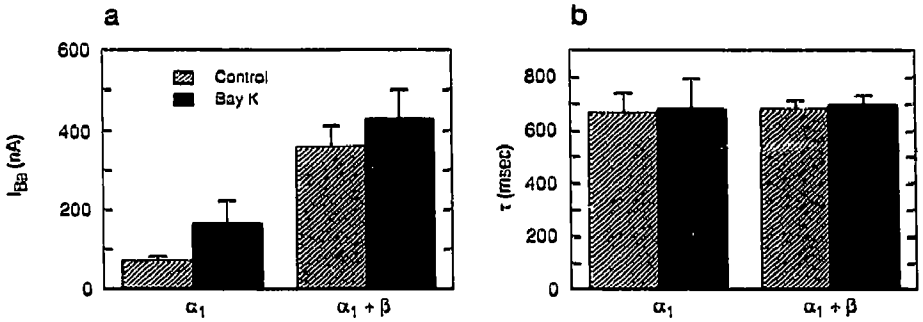

Fig. 3. Peak current amplitudes and inactivation time constants. (a) The peak current amplitudes, induced by VSM-α_1 and by co-injection of SK-β. The hatched and closed columns are the amplitudes before and after the addition of 10^{-6} M Bay K 8644, respectively. The mean ± S.E.M. are indicated. The number of experiments are 4 and 16 for VSM-α_1 and co-injection of SK-β, respectively. (b) Inactivation time constants (τ) of VSM-α_1 and co-injection of SK-β. The hatched and closed columns are before and after the addition of 10^{-6} M Bay K 8644, respectively. The mean ± S.E.M. are indicated. The number of experiments are 4 and 16 for VSM-α_1 and co-injection of SK-β, respectively.

suppressed by 10^{-6} M diltiazem (Fig. 2a) and by (±) PN 200-110 (data not shown). Fig. 2b shows the *I–V* curve of peak I_{Ba} of a VSM-α_1 and SK-β co-injected oocyte. The pattern of this *I-V* curve was similar to that of the VSM-α_1 injected oocytes. The peak I_{Ba}'s at 0 mV of −71±11 nA and −357±55 nA were enhanced by the addition of Bay K 8644 to levels of −165±56 nA and −429±73 nA for VSM-α_1 alone (mean± S.E.M., n=4) and VSM-α_1 co-injected with SK-β (mean± S.E.M., n=16), respectively (Fig. 3a). The effect of Bay K 8644 seems to be relatively small in VSM-α_1 and SK-β co-injected oocytes. This is due to the fact that some oocytes showed more than a 2-fold increase of current amplitude by the addition of Bay K 8644, while others showed a decrease of current amplitude, probably due to desensitization. The inactivation time constant (τ) was not significantly changed either by co-injection of SK-β or by the addition of Bay K 8644. τ's of 664±78 ms and 679±118 ms became 678±34 ms and 695±38 ms after the addition of Bay K 8644 for VSM-α_1 alone (mean ± S.E.M., n=4) and for VSM-α_1 co-injected with SK-β (mean± S.E.M., n=16), respectively (Fig. 3b). Comparable activation and inactivation time courses were reported for smooth muscle cells of rat vas deferens and rat mesenteric artery, using Ba^{2+} as a carrier

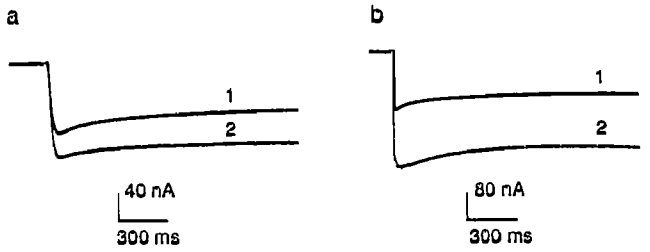

Fig. 4. I_{Ba} induced by DSHT-α_1 and co-injection of SK-α_2. (a) The current induced by DSHT-α_1 (trace 1) was enhanced by 10^{-6} M Bay K 8644 (trace 2). Cd^{2+}-insensitive currents were subtracted. (b) The current induced by co-injection of DSHT-α_1 and SK-α_2 (trace 1) was enhanced by 10^{-6} M Bay K 8644 (trace 2). Cd^{2+}-insensitive currents were subtracted.

[19,20]. Thus, our results suggest that the calcium channel expressed by α_1 alone or by α_1 plus β is similar to a native vascular smooth muscle calcium channel.

The I_{Ba} expressed by DSHT-α_1 (trace 1, Fig. 4a) was similar to the expressed VSM-α_1. The peak I_{Ba} of −80 nA at 0 mV was enhanced to −180 nA by the addition of 10^{-6} M Bay K 8644 (trace 2, Fig. 4a). The average increase of the current amplitude by the addition of 10^{-6} M Bay K 8644 was 2.3-fold (n=4). Co-injection of DSHT-α_1 and SK-α_2 in the same batch of oocytes resulted in a 2-fold increase in peak I_{Ba} compared to DSHT-α_1 alone injected oocyte (trace 1, Fig. 4b). The average increase of the peak current amplitude was 1.8-fold (n=4). Addition of 10^{-6} M Bay K 8644 in DSHT-α_1 and SK-α_2 co-injected oocytes increased the peak current amplitude 2.3-fold (n=4). An even larger increase was observed by co-injection of DSHT-α_1 and SK-β, i.e. by a factor of 4.8 (n=7) (data not shown). The increase of peak I_{Ba} was similar to that obtained by co-injection of VSM-α_1 and SK-β. Co-injection of either DSHT-α_1, or VSM-α_1, with SK-β and SK-α_2 resulted in still further increases in current density (data not shown). Analysis of the inactivation time course of these expressed currents did not reveal any major differences, compared to VSM-α_1 or to DSHT-α_1 alone expressed currents.

The fact that the expressed currents were greatly enhanced by the DHP calcium agonist Bay K 8644 and inhibited by Cd^{2+}, diltiazem or (±) PN 200-110 suggests that the currents expressed by VSM-α_1 and DSHT-α_1 are indeed of the L-type VDCC's. VSM-α_1 is homologous to DSHT-α_1 [12] which was expressed in oocytes here and the rabbit cardiac α_1-cDNA expressed by Mikami et al. [11]. These three clones all express Bay K 8644-sensitive currents with similar amplitudes. Sequence heterogeneity, however, does exist in two specific regions within VSM-α_1 and the cardiac-α_1 cDNA's [14] which may be of importance in the in vivo regulation of these two types of cardiovascular VDCC's. As we have reported [14], the third membrane-spanning

domain within the last motif (IVS3) of these α_1 clones differ; however, we have found that both forms (IVS3A and IVS3B) exist in rat aorta and rabbit heart. These two forms are apparently not tissue-specific as they have been observed in aorta, heart, and brain [15]. The cardiac isoform first described by Mikami et al. corresponds to IVS3A while the isoforms described here each harbor IVS3B. In this regard, both cardiac α_1 isoforms containing IVS3A or IVS3B as well as VSM-α_1 have been expressed and their currents appear to be similar. However direct comparative functional analyses need to be carried out to determine if these isoforms are functionally different. Interestingly, we have shown that these two distinct S3 regions arise from alternatively spliced adjacent exons of a single transcript [15]. A second region of diversity between the aortic and cardiac cDNA's is in a region which may play a role in the known distinct regulation patterns of these two channels, since it occurs in an area located in the intracellular COOH terminal region which is rich in potential phosphorylation sites [14]. While in vivo these two tissues (heart and VSM) display similar VDCC voltage sensitivity [20], they differ in their DHP sensitivity [20]. In addition VSM-VDCC may not be regulated by protein kinase A phosphorylation as is the case for the VDCC in cardiac tissue [2,3,20]. In this report, our oocyte data yields similar currents expressed from aorta and heart as well as calcium antagonist pharmacology. Experiments are ongoing to determine whether this second COOH area of diversity plays a role in channel regulation by other systems including phosphorylation.

It is now clear that the α_1-subunit alone does indeed encode a VDCC protein; the exact role of the smaller subunits remains to be determined. Co-injection of the skeletal muscle α_2 [9] cRNA with the cardiac [this paper, 11] enhanced the current amplitude but did not appear to change kinetics. Singer et al. [21] reported a 5-fold faster inactivation when cardiac α_1 was co-injected with skeletal α_2. However, there may be a significant difference between our α_1 and theirs, because their α_1 expressed current amplitude (5 nA) is considerably smaller than ours and that reported by Mikami et al. (around 80 nA). At this time, we have no other explanation for the disparate results.

We report here a significant enhancement of VSM-α_1 and DSHT-α_1 currents by the co-injection of skeletal β-subunit (SK-β). Therefore, the β subunit produces the same effect as the α_2 subunit does for cardiac α_1. Mori et al. [22] have shown that co-injection of SK-β cRNA enhanced the current expressed by a brain-specific DHP-insensitive α_1 cDNA, but did not change kinetics.

This study along with the above-mentioned experiments suggest a role for the α_2- and β-subunit in α_1 VDCC activity, but we emphasize that in each of these studies the co-expressions were with heterologous tissue cDNA's. Using a homologous expression system in which the SK-β was co-transfected with the skeletal muscle α_1 subunit in a mammalian cell line, we have shown that the SK-β enhances SK-α_1 kinetics but not current density and dramatically increases DHP binding [23]. In this respect, the skeletal VDCC is dissimilar to the cardiac and vascular smooth muscle VDCC. Underscoring the latter is the unresolved enigma that skeletal α_1 does not express in the oocyte system. In the present work we could not detect a significant change of the inactivation time constant, although we did see a significant enhancement of the current amplitude by the co-injection of SK-β. Although there is no direct evidence for the existence of a β-subunit in VSM, it is likely that all of the subunits do in fact exist in all tissues possessing VDCC activity and they may play a role in the regulation of the channel [24]. It is clear, though, that the major differences produced by homologous vs. heterologous expression need to be resolved.

The major point of the present study is that unlike skeletal α_1 expressed in mammalian cells, the cardiac and aorta α_1 subunits express a native-like calcium current in oocytes that is enhanced by β and α_2 subunits.

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