Regulation of the cardiac L-type calcium channel in L6 cells by arginine-vasopressin

Basil M. HANTASH¹, Andrew P. THOMAS and John P. REEVES

Department of Pharmacology and Physiology, Graduate School of Biomedical Sciences, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103, U.S.A.

L-type Ca^{2+} channel activity was measured in L6 cells as nifedipine-sensitive barium (Ba^{2+} ; 5 mM) influx in a depolarizing salt solution containing 140 mM KCl. Addition of AVP (arginine-vasopressin) during Ba^{2+} uptake reduced the rate of Ba^{2+} influx by 60–100%; this was followed by a gradual restoration of the initial rate of Ba^{2+} uptake. Blockade of PKC (protein kinase C) by pretreatment with 10 μM bisindolylmaleimide did not affect the initial inhibition of Ba^{2+} influx, but completely abolished the recovery phase. The effect of AVP was half-maximal at 10 nM AVP and was blocked by the V1a receptor antagonist d-(CH_2)₅-Tyr(Me)-AVP. Activation of G_{us} by isoprenaline or cholera toxin antagonized the actions of AVP on Ba^{2+} uptake. This protection

persisted in the presence of the PKA (protein kinase A) inhibitor KT5720, and was not mimicked by agents that increase cAMP. Inhibition of Ba^{2+} influx was also elicited by ATP and ET (endothelin 1) with an order of effectiveness $\text{ET} < \text{ATP} < \text{AVP}$. Each of these agents has been reported to act through G_q -coupled receptors. We conclude that activation of G_q -coupled receptors produces a rapid inhibition of the cardiac L-type Ca^{2+} channel, which is subsequently overcome by activation of PKC.

Key words: arginine-vasopressin (AVP), cardiac L-type calcium channel, G-protein, L6 cell, protein kinase C (PKC).

INTRODUCTION

The voltage-dependent Ca^{2+} channels of the long-lasting, or L-type, play important roles in a variety of cellular functions, including for example the coupling of excitation and contraction in the heart [1]. Regulation of Ca^{2+} influx through L-type Ca^{2+} channels allows control of the strength of contraction as well as the rate of heartbeat [2]. The cardiac L-type Ca^{2+} channel is a heteromultimeric complex composed of three unique subunits: a pore-forming and voltage-sensing α_1 , a predominantly extracellular $\alpha_2\delta$, and a cytosolically disposed β subunit (reviewed in [3]). The functions of the auxiliary subunits are less well understood, but clearly include a role in regulation of channel activity [3].

In addition to the auxiliary subunits, numerous protein kinases have been shown to modulate channel activity [3]. These protein kinases are often activated in response to hormones or neurotransmitters binding their respective receptors. For example, PKC (protein kinase C) and PKA (protein kinase A) are activated downstream of engagement of G-protein-coupled receptors, such as the AVP (arginine-vasopressin) receptor. AVP exerts both positive and negative inotropic effects on the cardiovascular system by acting on at least two types of receptors, known as V1a and V2. Both receptors are G-protein-coupled; however, V1a receptors lead to PLC (phospholipase C) stimulation, while V2 receptors increase AC (adenylate cyclase) activity.

Several studies have examined the role of AVP in modulation of L-type Ca^{2+} channel activity, with conflicting results. In the rat aortic smooth-muscle cell line (A7r5), Galizzi et al. [4] reported that treatment with AVP, oxytocin or bombesin for several minutes significantly reduced L-type Ca^{2+} channel activity, measured as $^{45}\text{Ca}^{2+}$ uptake, under depolarizing conditions or after activation by Bay K 8644. They concluded that the effects of AVP were

mediated by either PKC or a rise in $[\text{Ca}^{2+}]_{\text{c}}$ (cytosolic Ca^{2+} concentration). On the contrary, Marks et al. [5] found no effect of AVP or PMA on L-type Ca^{2+} currents ($I_{\text{Ca,L}}$) in the same cell line. In guinea-pig ventricular myocytes, Bonev and Isenberg [6] demonstrated an AVP-induced enhancement of $I_{\text{Ca,L}}$ that was not mimicked by addition of 8-bromo-cAMP, 8-bromo-cGMP or PMA. In all of the above studies, the AVP effect was always slow (minutes) in onset, suggesting the involvement of diffusible second messengers. Taken together, these studies reveal that the mechanism by which AVP modulates $I_{\text{Ca,L}}$ may be more complicated than initially hypothesized.

In the present study, we used L6 cells, a myogenic cell line established from newborn rat thigh muscle [7] to examine L-type Ca^{2+} channel activity. L6 cells in the myoblast stage of development express the cardiac isoform of the voltage-gated sodium channel [7] and we have recently found that they also express the $\alpha_1\text{C}$ (cardiac) form of the L-type Ca^{2+} channel; we could not detect the skeletal-muscle $\alpha_1\text{S}$ form of the channel (B. M. Hantash, A. P. Thomas and J. P. Reeves, unpublished work). They express a wide variety of G-protein-linked receptors, making them a useful model for investigating the influence of receptor agonists on L-type channel activity. L6 cells are readily grown in tissue culture, and after fura 2 loading, they can be used to measure L-type Ca^{2+} channel activity as depolarization-induced, nifedipine-sensitive Ba^{2+} influx. Here, we investigated the effects of G_q activation through AVP on L-type Ca^{2+} channel activity in L6 rat skeletal myoblasts. Under conditions where internal Ca^{2+} stores were depleted by prior treatment with thapsigargin and ionomycin, we found that AVP inhibited Ba^{2+} uptake immediately following its addition to the cells. Recovery of the rate of Ba^{2+} influx was observed within 60 s after addition of AVP. The recovery phase, but not the initial AVP-induced inhibition, was

Abbreviations used: AC, adenylate cyclase; AVP, arginine-vasopressin; CHX, cycloheximide; CTX, cholera toxin; $[\text{Ca}^{2+}]_{\text{c}}$, cytosolic Ca^{2+} concentration; DAG, diacylglycerol; ET, endothelin 1; $I_{\text{Ca,L}}$, L-type Ca^{2+} currents; IP_3 , inositol 1,4,5-trisphosphate; PI3K , phosphoinositide 3-kinase; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; PSS, physiological saline solution; PTX, pertussis toxin; P2Y, type 2Y purinergic; r.u., ratio units.

¹ To whom correspondence should be addressed, at Stanford University School of Medicine, Department of Surgery, Division of Plastic Surgery, 257 Campus Drive, Stanford, CA 94305, U.S.A. (email bhantash@stanford.edu).

blocked by PKC inhibitor. Activation of $G_{\alpha s}$ protected against the actions of AVP; this effect was not dependent on AC or PKA activity. The inhibitory effects of AVP are consistent with a membrane-delimited process, perhaps involving G-proteins, which may directly couple the V1a receptor and dihydropyridine-binding sites, as suggested by Grazzini et al. [8].

EXPERIMENTAL

Cell culture

L6 rat skeletal myoblasts were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 100 units/ml penicillin, 100 μ g/ml streptomycin and 10 μ g/ml gentamicin at 37°C in a 5 % CO_2 humidified atmosphere. Cells were subcultured every 3–4 days.

Reagents

Bisindolylmaleimide, 3-isobutyl-1-methylxanthine, wortmannin, U-73122, U-73343 and KT5720 were obtained from Calbiochem (La Jolla, CA, U.S.A.); D609 $\cdot K^+$, PMA and forskolin were from Alexis Biochemicals (San Diego, CA, U.S.A.); CTX (cholera toxin) and PTX (pertussis toxin) were from List Biological Laboratories (Campbell, CA, U.S.A.); SK&F 96365 was from Biomol (Plymouth Meeting, PA, U.S.A.), and all other chemicals were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise indicated. CTX, PTX, D609, ATP, ET (endothelin 1) and AVP were dissolved in distilled deionized water. U-73122 and U-73343 were reconstituted in chloroform. All other drugs were dissolved in DMSO as concentrated stocks and diluted at least 1000-fold into the appropriate medium. This yielded a final DMSO or chloroform concentration of 0.1 % or less, which alone had no effect on L-type Ca^{2+} channel activity in our assay.

ADP-ribosylation and PKC down-regulation

In order to ADP-ribosylate all G-protein α subunits of the G_i family, we treated L6 cells for 18–24 h with 100 ng/ml PTX. PTX was also included in the final resuspension medium [Na-PSS [physiological saline solution; 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose and 20 mM Mops, buffered to pH 7.4 (37°C) with Tris] + 1 mM $CaCl_2$ + 1 % BSA] at the same concentration. CTX (1 μ g/ml) was substituted for PTX in order to ADP-ribosylate all G-protein α subunits of the G_q family. PKC down-regulation was achieved by treatment of cells with 1 μ M PMA for 18–24 h.

Fura 2 measurements

Cells were grown to confluence in 75 cm^2 polystyrene flasks and then washed twice with nominally Ca^{2+} -free Na-PSS. Depolarizing medium was prepared by substituting 135 mM KCl for 140 mM NaCl. Cells were detached by treatment with 0.25 % trypsin and then resuspended in Ca^{2+} -free Na-PSS, centrifuged for 2 min at 300 g, and resuspended in Na-PSS + 1 mM $CaCl_2$. Cells were again centrifuged and finally resuspended in an appropriate volume of Na-PSS + 1 mM $CaCl_2$ + 1 % BSA. Aliquots (300 μ l) of cells were loaded for 30 min with 3 μ M fura 2/AM (fura 2 acetoxymethyl ester; Invitrogen, Carlsbad, CA, U.S.A.) and treated with 250 μ M sulfinpyrazone (to retard fura 2 transport out of the cells). Once loaded, the cells were centrifuged for several seconds and resuspended in Na-PSS containing 0.3 mM EGTA, 1 μ M thapsigargin and 2 μ M ionomycin to deplete internal Ca^{2+} stores. In experiments designed to measure the release of Ca^{2+} from

internal stores, cells were instead resuspended in Na-PSS + 1 mM $CaCl_2$. In some cases, as indicated in the Figure legends, the drug addition was made 30 min prior to fura 2. After 5 min, cells were placed in a fluorescence cuvette containing 3 ml of depolarizing medium with or without drug treatment and changes in fluorescence were monitored at alternate λ_{ex} of 350 and 390 nm, with emission collected at 510 nm in a Photon Technology International (Birmingham, NJ, U.S.A.) RF-M 2001 fluorometer. When measuring the release of Ca^{2+} from internal stores, cells were instead placed in a cuvette containing 3 ml of Na-PSS + 0.3 mM EGTA, and fluorescence was monitored at alternate λ_{ex} of 340 and 380 nm and emission was collected at 510 nm. Ba^{2+} uptake or agonist-induced release of Ca^{2+} from internal stores was initiated at 30 s. All fluorescence values were corrected for autofluorescence determined for each set of experiments using unloaded cells. All experiments were performed at 37°C.

Data analysis

Time plots are presented for n number of experiments as mean values $\pm$ S.E.M. (error bars shown in Figures). Significance testing was carried out using Student's t test (two-tailed) for unpaired samples using the raw rate values unless otherwise indicated. Since internal Ca^{2+} stores had been depleted, the rate of Ba^{2+} uptake represented entry through both L-type and Ca^{2+} -release-activated Ca^{2+} channels. Nifedipine (1 μ M) was used to selectively block entry through L-type channels [8]. This allowed us to determine the contribution of these two channels to the total measured signal. These two components were classified as nifedipine-sensitive or -insensitive, with the latter including Ba^{2+} entry through Ca^{2+} -release-activated Ca^{2+} channels and other passive leak pathways. Note that ionomycin does not transport Ba^{2+} at the concentrations used in our experiments [8]. We found that the nifedipine-insensitive component contributed 24 ± 2 and 17 ± 2 % of the total signal measured from 35–65 and 65–95 s respectively. The rates of nifedipine-sensitive Ba^{2+} influx were therefore computed by multiplying the raw rates by 0.76 or 0.83, as appropriate. In experiments where PKC was down-regulated, the total rate of Ba^{2+} influx was reduced by approx. 36 % compared with controls. The nifedipine-insensitive Ba^{2+} influx was not affected by PKC down-regulation and therefore comprised a greater proportion (38 ± 3 %) of the total Ba^{2+} uptake. For these experiments, we subtracted out the nifedipine-insensitive rate value obtained above from all the raw rate values to arrive at the nifedipine-sensitive component. Histogram plots are displayed for n number of experiments as the means $\pm$ S.E.M. calculated using the corrected nifedipine-sensitive rate values. We believe that this value is a more accurate representation of the rate of Ba^{2+} uptake through L-channels.

RESULTS

Inhibition of cardiac L-type Ca^{2+} channel activity by vasopressin

To investigate the role of AVP in regulation of the cardiac L-type channel in L6 cells, we first confirmed the presence of functional V1a receptors. As reported previously [9], addition of AVP (100 nM) to suspensions of fura 2-loaded L6 cells in a Ca^{2+} -free medium caused a rapid rise in cytosolic Ca^{2+} concentration due to release of IP_3 (inositol 1,4,5-trisphosphate)-sensitive internal Ca^{2+} stores (results not shown). In the following experiments, we depleted intracellular Ca^{2+} stores by pre-incubating cells with the Ca^{2+} ionophore ionomycin and the endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin, to avoid changes in L-type channel activity due to an increase in $[Ca^{2+}]_i$. Channel activity was then

Figure 1 Vasopressin-mediated inhibition of Ba^{2+} uptake by the L-type Ca^{2+} channel

(A) Cells were pre-incubated for 5 min in Na-PSS containing 0.3 mM EGTA, 1 μM thapsigargin and 2 μM ionomycin to deplete internal Ca^{2+} stores. Cells were then transferred to a cuvette containing depolarizing medium. Trace 'C' represents the control. For trace 'A', 100 nM AVP was added at 30 s along with 5 mM Ba^{2+} (arrow). (B) Same experiment as in (A) except that 10 μM bisindolylmaleimide (traces labelled 'B' and 'B + A') was added to the cells 30 min prior to loading with fura 2. Traces represent means $\pm$ S.E.M. for five individual experiments ($P < 0.005$).

measured as Ba^{2+} uptake. It should be noted that ionomycin itself does not induce Ba^{2+} uptake [10].

As illustrated in Figure 1(A), addition of AVP to the cuvette during Ba^{2+} uptake caused a transient decline in the rate of Ba^{2+} influx, followed by a restoration of the initial rate of Ba^{2+} uptake. An initial small increase in the fura 2 signal is due to extracellular fura 2; following the initial increase, the rate of Ba^{2+} influx was reduced by 76%. The rapid inhibition of Ba^{2+} influx by AVP was not due to release of Ca^{2+} from internal stores and subsequent Ca^{2+} -dependent inactivation, since even a supra-maximal concentration of AVP did not elicit a Ca^{2+} transient in these ionomycin-treated cells (results not shown). A nifedipine-insensitive component of Ba^{2+} influx, comprising $24 \pm 2\%$ ($n = 11$) of the total, was observed in these experiments and probably reflects store-operated Ca^{2+} entry, since it was abolished by 50 μM SK&F 96365 [11], a blocker of store-operated Ca^{2+} channels. The nifedipine-insensitive component was not affected by treatment with AVP (results not shown). Thus the effects of AVP on Ba^{2+} uptake in L6 cells are due exclusively to inhibition of the cardiac L-type Ca^{2+} channel.

To examine the role of PKC in the effects of AVP, we pre-incubated cells with bisindolylmaleimide, a selective PKC inhibitor. As demonstrated in Figure 1(B) (trace 'B'), this treatment reduced the initial rate of Ba^{2+} uptake by $34 \pm 6\%$ ($n = 5$, $P < 0.005$). Bisindolylmaleimide was without effect on the nifedipine-insensitive Ba^{2+} uptake (results not shown), indicating that this treatment reduced Ba^{2+} entry specifically through the L-type channel. Figure 1(B) also illustrates that AVP inhibited Ba^{2+} influx in bisindolylmaleimide-treated cells (trace 'B + A') to the same extent as for control cells; the inhibition of the nifedipine-sensitive rate of Ba^{2+} influx was 88 ± 4 and $85 \pm 1\%$ respectively ($n = 5$, $P > 0.5$). This result demonstrated that the initial inhibition was not mediated by PKC. However, the recovery phase, measured as the rate of Ba^{2+} influx from 65 to 95 s, was completely abolished by pre-incubation with bisindolylmaleimide [0.007 r.u./s (ratio units/s) for AVP versus 0.001 r.u./s for

Figure 2 PKC mediates recovery from inhibition of Ba^{2+} influx by AVP

(A) Cells were assayed as in Figure 1(A). Ba^{2+} (5 mM) was added to the cuvette at 30 s with (A) or without (C) 100 nM AVP. (B) Same experiment as (A) except that PKC was down-regulated by treating cells with 1 μM PMA for 18–24 h. Results represent means $\pm$ S.E.M. for five or six independent runs ($P \leq 0.01$).

Figure 3 V1a receptor antagonist prevented the effect of AVP on the L-type Ca^{2+} channel

Cells were treated as in Figure 1(A) except that ionomycin was omitted from the pre-incubation. 1 μM of the V1a receptor antagonist d-(CH₂)₅ Tyr(Me)-AVP (traces T and T + A) was added to the cuvette just before transfer of the cells. Then, 100 nM AVP (traces A and T + A) or no treatment (trace C) was added to the cuvette 30 s after Ba^{2+} uptake was initiated. Rates of Ba^{2+} uptake were taken from 65 to 95 s. Results shown are means $\pm$ S.E.M. for five or six separate experiments ($P < 0.05$). For clarity, trace 'T' is in black and all traces lack error bars.

bisindolylmaleimide + AVP; $n = 5$, $P < 0.02$). Similar results were obtained when cells were exposed to PMA overnight to down-regulate PKC. As shown in Figure 2(B) (trace 'C'), this treatment alone reduced the basal uptake rate by approx. 36% compared with control conditions (Figure 2A, trace 'C'). To determine whether PKC affected the nifedipine-sensitive or -insensitive component, we compared the nifedipine-insensitive uptake rates before and after down-regulation of PKC. The nifedipine-insensitive Ba^{2+} influx rate in PKC down-regulated cells (0.008 r.u./s, $n = 7$) was identical with that of control cells (0.008 r.u./s, $n = 11$; results not shown). Figure 2(B) (trace 'A')

also shows that AVP further inhibited the initial rate of Ba^{2+} entry by $93 \pm 3\%$ ($n = 6$, $P = 10^{-5}$) relative to untreated cells. Finally, as expected, down-regulation of PKC completely blocked recovery (compare trace 'A' in Figures 2A and 2B, from 90 to 250 s).

To test the possibility that the effect of AVP may be mediated by a different serine–threonine protein kinase, we performed a similar set of experiments substituting staurosporine, a non-selective cell-permeable protein kinase inhibitor, for bisindolylmaleimide. As with bisindolylmaleimide, the initial inhibition by AVP was not modified by prior treatment with staurosporine, although the recovery phase was blocked (results not shown). In addition, staurosporine alone, similar to bisindolylmaleimide, caused a $34 \pm 9\%$ ($n = 4$) inhibition of Ba^{2+} uptake. Thus these results suggest that the initial AVP-induced inhibition is probably not mediated by a serine–threonine protein kinase, although the recovery phase is PKC-dependent. Finally, the fact that bisindolylmaleimide and PKC down-regulations both suppress basal L-type channel activity suggests that channel activity is enhanced by PKC under basal conditions in untreated cells. Data providing further support for this conclusion will be published elsewhere.

Characterization of the AVP-mediated effects on Ca^{2+} channels

Since AVP can act through V1a, V1b or V2 receptors, we next examined whether the effects of AVP on cardiac L-type Ca^{2+} channel activity were a result of activation of one or more of these receptors. Figure 3 shows that addition of AVP (100 nM) 30 s after initiation of Ba^{2+} uptake immediately and completely abrogated further influx of Ba^{2+} during the ensuing 60 s. This was followed by a time-dependent restoration of the uptake rate to one that approximated that seen for the control. The data in Figure 3 show that the effects of AVP were essentially eliminated by pre-incubating cells with the competitive and selective V1a receptor antagonist d-(CH_2)₅-Tyr(Me)-AVP (1 μM), which alone had no significant effect on Ba^{2+} influx [12,13]. Furthermore, treatment with the cell-permeable cAMP analogue dibutyryl-cAMP (1 mM) had no effect on Ba^{2+} influx, precluding the possibility that the rapid inhibition by AVP was due to activation of the V2 receptor and consequent production of cAMP (results not shown). We conclude that modulation of cardiac L-type Ca^{2+} channel activity by AVP is mediated exclusively through V1a receptors.

As indicated in Figure 4, AVP displayed a concentration-dependent effect with half-maximal inhibition at approx. 10 nM, achieving a maximal inhibition of approx. 85% at 100 nM. The residual component of Ba^{2+} undoubtedly reflects the activity of store-operated channels, which are unaffected by AVP (see above). This concentration dependence is similar to that obtained by Gardner et al. [10], who reported an EC_{50} of 3 nM and maximal effect at 100 nM for AVP-mediated stimulation of inositol phosphate generation in L6 myoblasts.

Activation of G_q -coupled receptors modifies cardiac L-type Ca^{2+} channel activity

The V1a receptor has been extensively studied, and its coupling with G_q is well characterized [14,15]. Previously, a G-protein-mediated coupling between V1a receptors and dihydropyridine-binding sites was reported in rat glomerulosa cells. Thus the effect of AVP on the cardiac Ca^{2+} channel may represent a preferential coupling between the V1a receptor and the L-type Ca^{2+} channel. Alternatively, activation of G_q -coupled receptors, in general, may result in similar effects to those observed with AVP. To distinguish between these two possibilities, we took advantage of the fact that L6 cells also express ET(A) receptors [16], and muscle

Figure 4 Dose-dependent effect of AVP on L-type Ca^{2+} channel activity

(A) Cells were treated as in Figure 1(A) except that they were exposed to varying concentrations of AVP (100 pM to 300 nM) at 30 s. The initial rates of nifedipine-sensitive Ba^{2+} uptake, measured 30 s after the addition of Ba^{2+} , are displayed as a percentage of control. Means for four different trials are shown. The effect of AVP became significant at 3 nM (* $P < 0.01$).

Figure 5 ET and ATP induce release of internal Ca^{2+} stores in L6 cells

(A) Cells were pre-incubated for 5 min in Na-PSS + 1 mM CaCl_2 and then transferred to a cuvette containing Na-PSS + 0.3 mM EGTA. Trace shows the resultant change in $[\text{Ca}^{2+}]_i$ after addition of 300 μM ATP or 100 nM AVP at 30 s (depicted by arrow). (B) Same format as (A), except that 100 nM ET replaced ATP. Means $\pm$ S.E.M. for three to five individual experiments are shown.

cells endogenously express P2Y (type 2Y purinergic) receptors [17], both of which also couple with G_q . Activation of P2Y or ET(A) receptors with ATP (300 μM) or ET (100 nM) respectively resulted in the release of Ca^{2+} from intracellular stores (Figures 5A and 5B). Both ATP and ET were less effective than AVP at inducing the release of Ca^{2+} from internal stores as determined from the peaks of the Ca^{2+} transients. The order of effectiveness was $\text{ET} < \text{ATP} < \text{AVP}$ and probably correlates with the relative densities of their respective receptors on the plasma membrane.

Figure 6 shows that addition of ATP or ET during Ba^{2+} uptake attenuated Ba^{2+} influx by 44 ± 12 or $23 \pm 4\%$ respectively compared with 65 ± 3 or $66 \pm 4\%$ for AVP ($n = 5$, $P < 0.05$). These data do not support the notion that V1a receptors selectively couple with cardiac L-type channels in L6 cells. These results suggest a more general interaction between G_q -coupled receptors and cardiac L-type Ca^{2+} channels rather than a preferential coupling between V1a receptors and dihydropyridine-binding sites.

Figure 6 ET- and ATP-mediated inhibition of L-type Ca²⁺ channel activity

L6 cells were treated as in Figure 1(A). (A) Addition of 300 μ M ATP (ATP), 100 nM AVP (A) or no treatment (C) was made at the same time as BaCl₂. (B) Same experiment as in (A), except that 100 nM ET (E) replaced ATP. Histograms plotting the initial uptake rates of data taken from (A, B). Results represent the means $\pm$ S.E.M. for three to five different experiments (* $P < 0.05$).

Figure 7 U-73122 reduces Ca²⁺-store release by AVP without affecting AVP-mediated inhibition of Ba²⁺ uptake

(A) Cells were pretreated as in Figure 5(A), but in the presence (trace U) or absence (trace C) of 4 μ M U-73122. AVP (100 nM) was added at 30 s. (B) Cells were handled as in Figure 1(A), except that 4 μ M U-73122 (traces U and U + A) or no addition (traces C and A) was included in the 5 min pre-incubation. Then, 100 nM AVP (traces A and U + A) was added at the same time as Ba²⁺. Initial uptake rates are displayed as a histogram. Means $\pm$ S.E.M. for six determinations from four separate experiments were calculated. No significant difference was found between traces 'A' and 'U + A' ($P > 0.4$).

PLC does not mediate inhibition of L-type Ca²⁺ channel activity by AVP

Activation of G_q-linked receptors leads to stimulation of PLC β and hydrolysis of PIP₂ (phosphatidylinositol 4,5-bisphosphate) into IP₃ and DAG (diacylglycerol). Our results above (see Figure 1) suggested that neither release of IP₃-dependent intracellular Ca²⁺ stores nor DAG-dependent activation of PKC could account for the rapid inhibition of Ba²⁺ uptake by AVP. To assess whether PLC might nonetheless be involved in the effects of AVP, we inhibited PLC activity with the aminosteroid U-73122 [18]. Since U-73122 itself decreased Ba²⁺ influx in these cells, it was necessary to use a low concentration of this agent. We chose 4 μ M U-73122, a concentration that reduced Ba²⁺ entry by $25 \pm 5\%$ (Figure 7B; $n = 6$, $P < 0.01$). Figure 7(A) illustrates

Figure 8 Inhibition of Ba²⁺ uptake by AVP is PTX-insensitive but CTX-sensitive

(A) Cells were exposed to 100 ng/ml PTX for 18–24 h and then processed similar to Figure 1(A). 100 nM AVP (A) or no treatment (C) was added at 30 s with BaCl₂. (B) Identical experiment as in (A), except that 1 μ g/ml CTX was substituted for PTX. Means $\pm$ S.E.M. for nine to eleven individual runs from four different experiments are shown ($P < 0.02$).

that a 5 min treatment with U-73122 led to a $>50\%$ inhibition of AVP-induced mobilization of Ca²⁺ from internal stores as measured by the peak of the Ca²⁺ transient. The inactive analogue U-73343 had no effect on AVP-mediated Ca²⁺ release (results not shown, [19]). The data in Figure 7(B) demonstrate that the inhibition of Ba²⁺ uptake by AVP was not significantly altered ($73 \pm 2\%$ for AVP versus $70 \pm 7\%$ for U-73122 + AVP; $n = 6$, $P > 0.4$) by the presence of U-73122. If PLC activity were directly involved in mediating the effects of AVP on channel activity, the AVP-mediated inhibition of Ba²⁺ influx would be reduced by U-73122. For example, the Ca²⁺ transient induced by ATP (Figure 5A) was comparable with that of AVP in the presence of U-73122 (Figure 7A). With U-73122, however, despite reducing PLC activity to the level seen with ATP, inhibition of Ba²⁺ uptake by AVP was unchanged, suggesting that the inhibitory effect was mediated by a signal upstream from PLC.

Previously, it has been reported that treatment with AVP leads to activation of phosphatidylcholine-specific PLC [20], PI3K (phosphoinositide 3-kinase) [21], as well as the tyrosine kinase Src [22]. We therefore repeated the experiments in Figure 6(B), substituting D609 (100 μ M), a potent inhibitor of phosphatidylcholine-specific PLC [23], wortmannin (100 nM), a selective inhibitor of PI3K at this concentration [24], or pyrazolopyrimidine (10 μ M), a specific inhibitor of tyrosine kinases [25], for U-73122. Even after these manoeuvres, the inhibition by AVP remained completely intact (results not shown). Altogether, it appears that AVP's inhibitory action is independent of PLC β , phosphatidylcholine-specific PLC, PI3K and tyrosine kinases.

Activation of G_{as} protects against inhibition by AVP

Recent studies indicate that G-protein-linked receptors may not always couple faithfully with G-proteins (see [26] for a review). Specifically, the V1a receptor has been shown to couple with a PTX-sensitive G-protein [8,27]. We therefore examined whether or not the inhibition by AVP we observed was mediated by a PTX-sensitive G-protein. Overnight treatment with PTX (100 ng/ml) did not affect coupling between V1a receptors and G_{aq}, since cells responded with a similar rise in [Ca²⁺]_i after challenge with AVP (100 nM) as untreated cells (results not shown). Figure 8(A) shows that in PTX-treated cells, AVP inhibited Ba²⁺ influx by $43 \pm 4\%$

Figure 9 Effect of CHX on CTX-mediated protection against AVP

Cells were incubated overnight with 10 μ M CHX with (B) or without (A) 1 μ g/ml CTX and then handled as in Figure 1(A). 100 nM AVP (A) or no addition (C) was included with 5 mM Ba²⁺ at 30 s. (C) Rates of Ba²⁺ uptake from 35 to 65 s were plotted for the data in (A, B). Results are presented for three separate cell preparations assayed in duplicate.

($n = 11$), a value somewhat less than that observed in untreated cells (60–100 %, Figures 1, 2A, 4, 6 and 7B). However, since the toxin-sensitive substrates would be fully ADP-ribosylated and thus inhibited under these conditions [10], the results suggest that the major effect of AVP is not mediated by PTX-sensitive G-proteins. The partial protection may be due to PTX-induced activation of AC (see below).

To examine the role of G_s proteins in the actions of AVP, we pre-incubated cells with CTX. Surprisingly, the effect of AVP was reduced to a modest 20 ± 6 % inhibition (Figure 8B; $n = 9$, $P < 0.02$). This protection was not observed when cells were acutely (5 min or 3 h) exposed to CTX, indicating that CTX did not act as antagonist of the V1a receptor (results not shown). Furthermore, as mentioned above, CTX-treated cells displayed normal Ca²⁺ transients upon challenge with AVP. Thus the above experiments suggested that CTX-dependent activation of G_{as} attenuates the AVP-mediated inhibition of Ba²⁺ uptake without altering coupling between AVP and its receptor. The effects of CTX were complicated by an increase in the basal rate of Ba²⁺ uptake (Figure 8B); however, Figure 9(A) shows that this increase was completely blocked by CHX (cycloheximide), suggesting that CTX increased L-type Ca²⁺ channel expression. Overnight incubation with CHX (10 μ M) in the presence or absence of CTX did not affect the AVP-induced Ca²⁺ transient, indicating that these treatments did not influence the number of V1a receptors expressed at the plasma membrane (results not shown). Cells treated with CHX or CHX + CTX displayed a similar basal Ba²⁺ influx rate as untreated cells (compare Figures 1A and 9, traces marked 'C'). As shown in Figure 9(A), CHX did not significantly affect the AVP-mediated inhibition of Ba²⁺ uptake (65 ± 5 %; $n = 6$, $P = 0.001$). CTX still protected against AVP-induced inhibition in CHX-treated cells (34 ± 6 % inhibition for CHX + CTX versus 65 ± 5 % inhibition for CHX alone; $n = 6$, $P < 0.005$).

The above experiments involved prolonged treatments (18–24 h) with the G_{as} activator CTX, and a multitude of changes may take place during this time. Thus we took advantage of the fact that L6 cells express β_2 -adrenergic receptors [28], allowing us to activate G_{as} acutely without the possibility of inducing changes in cellular expression patterns. Figure 10 illustrates that cells exposed for 5 min to the β_2 -adrenergic receptor agonist

Figure 10 Isoprenaline protects against the effect of AVP on L-type Ca²⁺ channel activity

(A) Cells were treated similar to Figure 1(A), except that 20 μ M isoprenaline (traces I and I + A) or no addition (traces C and A) was included in the 5 min pre-incubation. Ba²⁺ uptake was initiated at 30 s with (traces A and I + A) or without (traces C and I) 100 nM AVP. (B) Rates of Ba²⁺ influx for the results shown in (A). The results are the means $\pm$ S.E.M. for five independent experiments. For traces 'I' and 'I + A', standard errors were not displayed for clarity.

isoprenaline (20 μ M) did not display a PKA-dependent increase in activity, but this short treatment was able to reduce the inhibition by AVP from 59 ± 7 to 18 ± 10 % ($n = 5$, $P < 0.05$). The isoprenaline protection was not altered by pretreatment with KT5720, a selective PKA inhibitor (results not shown). Similarly, KT5720 did not modify the CTX-mediated protection against AVP (results not shown). Furthermore, acute activation of PKA with forskolin and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine neither increased basal Ba²⁺ uptake nor modified the inhibitory effect of AVP (results not shown). Altogether, our results reveal a previously unsuspected yet profound inhibitory regulatory pathway downstream of G_q-coupled receptor activation which is independent of PLC, PKC or the release of internal Ca²⁺ stores. This negative modification of cardiac Ca²⁺ channel activity appears to be strongly antagonized by prior activation of the heterotrimeric G_s protein, but not PKA.

DISCUSSION

The effects of AVP on cardiac L-type channels have been extensively studied, with variable results. Treatment with AVP has been shown to stimulate [8], inhibit [4], or have no effect on $I_{Ca,L}$ [5]. Although no consensus has been reached about the direction of modulation of $I_{Ca,L}$ by AVP, both the stimulatory and inhibitory effects have been attributed to either activation of PKC or a rise in $[Ca^{2+}]_i$. In the present study, we measured L-type Ca^{2+} channel activity in L6 cells as the rate of Ba^{2+} influx in a depolarizing medium. Addition of AVP simultaneously with Ba^{2+} reduced the initial rate of Ba^{2+} influx by 60–100%; this was followed by a gradual restoration of Ba^{2+} uptake (Figure 1A). Inhibition or down-regulation of PKC did not lessen the inhibitory effects of AVP, although these treatments did block the subsequent recovery of channel activity (Figures 1 and 2; see below). To eliminate effects of changes in $[Ca^{2+}]_i$ in our experiments, we depleted internal Ca^{2+} stores with thapsigargin before exposing cells to AVP. Thus the inhibitory effects of AVP on L-type channel activity were independent of either PKC or changes in $[Ca^{2+}]_i$.

The effects of AVP influx were completely blocked by preincubating cells with a selective V1a receptor antagonist (Figure 3). We precluded the possibility that AVP was acting through V2 receptors by bypassing the receptor and increasing cytosolic cAMP levels with the cell-permeable analogue dibutyryl-cAMP. This treatment did not inhibit Ba^{2+} uptake (results not shown), confirming that the effects of AVP were mediated exclusively through the G_q -coupled V1a receptor. Moreover, a similar inhibition was observed when cells were challenged with either ET or ATP (Figure 6), both of which act through G_q -coupled receptors [16,17]. As this negative modulation was elicited by three different G_q -coupled receptors, our results are not in support of the previous notion that V1a receptors selectively couple with DHP (dihydropyridine)-binding sites [8]. Instead, we conclude that activation of G_q -coupled receptors, in general, leads to inhibition of cardiac Ca^{2+} channel activity in L6 cells. The rank order $ET < ATP < AVP$ of effectiveness of these agonist in inhibiting Ca^{2+} channel activity and evoking the $[Ca^{2+}]_i$ transient was the same, indicating that inhibition of channel activity was directly related to the degree of engagement of G_q -coupled receptors.

Although channel activity was initially inhibited by AVP, a gradual restoration of the rate of Ba^{2+} influx subsequently occurred, suggesting a time-dependent recovery of channel activity. This recovery was blocked by inhibiting or down-regulating PKC (Figures 1 and 2). We considered the possibility that this was due to a PKC-dependent desensitization of the V1a receptor or a PKC-promoted inhibition of PLC activity. However, we found no significant difference in the AVP-induced Ca^{2+} transient before or after PMA treatment (results not shown). These results are consistent with a previous report showing that PKC, PKA and Ca^{2+} did not mediate desensitization of the V1a receptor [29], and argue against a PKC-mediated reduction of PLC activity [30]. Thus the mechanism by which PKC alters the time-dependence of the AVP effects on Ba^{2+} uptake remains elusive. We speculate that PKC may directly phosphorylate the $\alpha 1C$, or perhaps a closely associated protein or channel subunit, and this overcomes AVP's inhibitory actions. An analogous phenomenon has been previously reported for voltage-dependent Ca^{2+} channels at presynaptic nerve terminals [31]. In this case, $G_{\beta\gamma}$ -induced inhibition is antagonized by PKC-dependent phosphorylation of the α_1 subunit domain I–II linker, a site which contributes to $G_{\beta\gamma}$ binding as well as PKC-mediated up-regulation of channel activity. This highly elegant form of cross-talk may represent a mechanism by which cells can integrate multiple extracellular signals.

Activation of G_q -coupled receptors directly leads to the generation of two second messengers, IP_3 and DAG [32], through stimulation of PLC activity. Direct effects of IP_3 or DAG on $I_{Ca,L}$ have been previously reported (reviewed in [3]). We examined this possibility by measuring the effect of AVP on Ba^{2+} influx after inhibiting the activity of PLC with U-73122. Under these conditions, U-73122 reduced the size of the Ca^{2+} transient by > 50% (Figure 7A). Thus we expected that U-73122 would partially attenuate the effect of AVP on Ba^{2+} uptake if PLC activity were involved. However, U-73122 did not significantly modify the extent of inhibition of Ba^{2+} influx evoked by AVP (Figure 7B). We therefore conclude that the inhibitory effects of AVP are mediated by a signal upstream from PLC.

Activation of V1a receptors with AVP has been shown to simultaneously activate a number of signalling pathways in addition to PIP_2 hydrolysis [20–22]. Our experiments with staurosporine did not support the involvement of a serine–threonine protein kinase (other than PKC) in mediating the inhibitory effects of AVP (results not shown). Moreover, we found no evidence that phosphatidylcholine-specific PLC, PI3K or Src facilitated the effects of AVP as blockade of these enzymes with selective inhibitors did not modify the AVP-induced inhibition of Ba^{2+} uptake (results not shown). Infidelity of receptor–G-protein coupling has been observed for the V1a receptor [8,27]. However, PTX did not significantly alter the AVP-mediated Ca^{2+} transient (results not shown), and reduced only slightly the AVP-dependent inhibition of Ba^{2+} uptake (Figure 8A). This modest reduction in the efficacy of AVP is unlikely to be due to promiscuous coupling between the V1a receptor and G-proteins, since previous studies in L6 cells clearly demonstrated that PTX-sensitive G-proteins are fully inhibited after overnight treatment with PTX [33]. Instead, the partial protection may be due to activation of AC by PTX, as inhibition of G_{α_i} by PTX can leave AC activity unchecked [34]. This is supported by our results with overnight forskolin treatment that showed a similar degree of protection against inhibition by AVP (results not shown). However, prolonged activation of AC may be required for this partial protection as acute (5 min) treatment with forskolin did not change the magnitude of inhibition induced by AVP (results not shown). Furthermore, consistent with a previous report [33], these data also suggest that the protective effect could not be explained by a PKA-dependent desensitization of the V1a receptor.

The role of G_s proteins was then examined by inhibiting G_{α_s} GTPase activity with CTX pretreatment [35]. ADP-ribosylation of G_{α_s} led to a significant attenuation of the inhibitory actions of AVP (Figure 8B). Moreover, we observed a similar protection when cells were acutely treated with isoprenaline (Figure 10). The effects of both CTX and isoprenaline appeared to be independent of PKA (results not shown) and could not be mimicked by agents that activate AC (see above). Similar but not identical results were reported by Delpech et al. [36], who found that ET inhibited $I_{Ca,L}$ enhanced by isoprenaline in a PKC- and Ca^{2+} -independent fashion. Our experiments with isoprenaline suggest that the attenuation of the AVP response was due to activation of G_{α_s} , and argue against the possibility that the effects of CTX were indirectly caused by a decreased expression of G_q proteins or V1a receptors. Furthermore, previous reports have shown that G_q mRNA levels are increased, not decreased, after prolonged treatment with CTX [37–39]. Thus the results with isoprenaline and CTX implicate the G_s protein in the protection against the inhibitory effects of AVP.

The above results raise the possibility that $G_{\beta\gamma}$ may mediate the protection against AVP evoked by CTX and isoprenaline. So far, several groups have investigated the possibility that $G_{\beta\gamma}$ directly binds to the cardiac L-type Ca^{2+} channel, but no evidence was

Figure 11 Cross-talk between different G-protein-coupled receptor pathways affecting cardiac L-type Ca^{2+} channel activity in L6 myoblasts

(A) Upon binding of G_s -coupled receptor (R_s) or G_q -linked receptor (R_q) by its respective ligands (L), the $\beta\gamma$ subunit ($\beta\gamma$) dissociates from the α subunit. The $\beta\gamma$ subunit of G_s then binds and sequesters free G-protein α_q (α_q), thereby preventing G_{aq} from binding and inhibiting the voltage-dependent calcium channel (VDCC). Binding of G-protein α_s subunit (α_s) to its unique interaction site on the VDCC results in increased channel activity and masking of the G_{aq} -binding site. (B) Alternatively, G_{as} and G_{aq} may compete for identical binding sites, resulting in an interaction that precludes the binding of the other. IP_3R , IP_3 receptor.

found for such an interaction [40,41]. Instead, $G_{\beta\gamma}$ may remain free to bind and sequester other G-protein α subunits. These considerations led us to propose that AVP inhibits L-channel activity in L6 cells in a membrane-delimited fashion, perhaps via a direct interaction between G_{aq} and the L-type Ca^{2+} channel. Although it has long been known that G_{as} directly enhances cardiac $I_{\text{Ca,L}}$ [42–44], it has yet to be established whether G_q can also regulate L-type Ca^{2+} channel activity in a membrane-delimited manner. The rapidity of the inhibition of Ba^{2+} uptake by AVP is consistent with such a mechanism. In cells treated with CTX or isoprenaline, $G_{\beta\gamma}$ would counteract this effect by binding to G_{aq} liberated by AVP, thereby preventing its interaction with the channel, and reducing the inhibition by AVP (Figure 11A).

A second possibility is that G_{aq} and G_{as} compete for binding of a similar G-protein interaction domain somewhere on the Ca^{2+} channel (Figure 11B). In this scenario, G_{as} may be bound to the Ca^{2+} channel in the basal state. Activation of the V1a receptor would cause the release of G_{aq} , leading to a competition for the same interaction site. Alternatively, G_{as} and G_{aq} may bind separate interaction sites. In this case, the presence of an interaction between G_{as} and the channel would preclude binding of G_{aq} to its interaction site, thereby preventing its negative modulatory effect on channel activity (Figure 11A). At this point, it is difficult to conclude with certainty which scenario represents the more likely

mechanism linking G_{as} and G_{aq} . These scenarios are highly speculative and require confirmation by electrophysiological studies.

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