Modulation of Cardiac Ca\(^{2+}\) Channel by G\(_q\)-activating Neurotransmitters Reconstituted in Xenopus Oocytes*

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Sharon Weiss, Thanh Doan†‡, Kenneth E. Bernstein†‡, and Nathan Dascal§

From the Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel and the Departments of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia 30322

L-type dihydropyridine-sensitive voltage dependent Ca\(^{2+}\) channels (L-VDCCs; \(\alpha_{1C}\)) are crucial in cardiovascular physiology. Currents via L-VDCCs are enhanced by hormones and transmitters operating via G\(_q\), such as angiotensin II (AngII) and acetylcholine (ACh). It has been proposed that these modulations are mediated by protein kinase C (PKC). However, reports on effects of PKC activators on L-type channels are contradictory; inhibitory and/or enhancing effects have been observed. Attempts to reproduce the enhancing effect of AngII in heterologous expression systems failed. We previously found that PKC modulation of the channel depends on \(\alpha_{1C}\) isoform used; only a long N-terminal (NT) isoform was up-regulated. Here we report the reconstitution of the AngII- and ACh-induced enhancement of the long-NT isoform of L-VDCC expressed in Xenopus oocytes. The current initially increased over several minutes but later declined to below baseline levels. Using different NT deletion mutants and human short- and long-NT isoforms of the channel, we found the initial segment of the NT to be crucial for the enhancing, but not for the inhibitory, effect. Using blockers of PKC and of phospholipase C (PLC) and a mutated AngII receptor lacking G\(_q\) coupling, we demonstrate that the signaling pathway of the enhancing effect includes the activation of G\(_q\), PLC, and PKC. The inhibitory modulation, present in both \(\alpha_{1C}\) isoforms, was G\(_q\)- and PLC-independent and Ca\(^{2+}\)-dependent, but not Ca\(^{2+}\)-mediated, as only basal levels of Ca\(^{2+}\) were essential. Reconstitution of AngII and ACh effects in Xenopus oocytes will advance the study of molecular mechanisms of these physiologically important modulations.

The cardiac voltage-dependent, dihydropyridine-sensitive L-type calcium channel (L-VDCC) is the main calcium channel in the heart, where it contributes to the plateau of the action potential and thereby promotes cardiac cell contraction (1). In smooth muscle cells, these channels regulate tonus and contraction (2, 3). Different hormones and transmitters, such as angiotensin II (AngII), bradykinin, acetylcholine (ACh), and norepinephrine, modulate the function of L-VDCC via G-proteins and protein kinases, profoundly affecting the function of the corresponding tissues (4).

AngII and ACh activate G\(_q\)-coupled receptors and are involved in cardiovascular function, regulation of blood pressure, and renal function (5, 6). In the heart, ACh inhibits L-VDCC via m2 muscarinic receptors and the subsequent activation of the G\(_i\) signaling cascade and inhibition of adenyl cyclase (1). However, in the smooth muscle, both AngII and ACh are potent vasoconstrictors that both induce Ca\(^{2+}\) release from intracellular stores and elevate intracellular Ca\(^{2+}\) concentration (7, 8). In heart and smooth muscle, AngII enhances Ca\(^{2+}\) channel currents (9–13). ACh has also been reported to increase L-type Ca\(^{2+}\) channel currents in smooth muscle, mainly via m3 muscarinic receptors, m3R (14–16).

Despite the clinical and physiological importance of the regulation of L-type Ca\(^{2+}\) channels by AngII and ACh, the molecular mechanisms remain poorly understood. The mechanism of AngII effect on L-type Ca\(^{2+}\) channels has been extensively studied but remains unclear and even controversial. Protein kinase C (PKC) is the most obvious and important mediator of AngII and ACh/m3R action. PKC is activated in native cells following AngII and ACh binding to G\(_q\)-coupled receptors (AngII receptor type 1, AT1R, and muscarinic receptors m3R and m1R, respectively). In mammals, PKC inhibitors block AngII-induced vasoconstriction (17–20). In cardiomyocytes and smooth muscle cells, the PKC activators phorbol esters and diacylglycerol mimic the effect of AngII, increasing force of contraction and Ca\(^{2+}\) influx (10, 21, 22), as well as Ca\(^{2+}\) currents via the L-type channel (12, 23–30). This enhancement is sometimes followed by a later reduction in the current (24, 25, 31). In some cases, only an inhibition of the current in response to PKC activation has been reported (32). In addition to PKC, protein tyrosine kinases (33, 34) and the G\(_{\beta\gamma}\) dimer, via activation of phosphoinositol-3-kinase (35), have been implicated as being potentially involved in the mediation of AngII effects.

A major obstacle in studying the molecular mechanisms of AngII and ACh modulations was that these modulations could not be reconstituted in heterologous expression systems. Bouron et al. (36) studied the modulation of human L-VDCC expressed in Xenopus oocytes and reported that following the administration of PMA, the dihydropyridine-sensitive \(I_{Ca}\) was inhibited. Oz et al. (37) reported a decrease in Ca\(^{2+}\) current in oocytes expressing \(\alpha_{1C}\) and AT1R following application of AngII, due to a Ca\(^{2+}\)-dependent mechanism. This effect was blocked by chelating Ca\(^{2+}\) or by depleting intracellular Ca\(^{2+}\) stores with thapsigargin.

The failure to reproduce AngII-induced Ca\(^{2+}\) channel en-
hancement may be related to the use of certain isoforms of α1C that are not modulated by PKC. In the rat and rabbit, two N-terminal (NT) isoforms of α1C (Ca2,1,1) are known, which probably represent variable splicing products of the same gene (38). These splice variants encode long- and short-NT α1C proteins, with variable initial segments of 46 and 16 amino acids (aa), respectively (38–41) (the total length of the cytosolic part of the NT region of α1C is ~154 aa in the long-NT α1C). Studies of the molecular mechanism of PKC modulation of the rabbit long-NT isoform, expressed in Xenopus oocytes, identified the first 46 aa as crucial for PKC modulation (42). This was further narrowed down to the first 5 aa being essential for PKC action (43). Recently, similar N-terminal isoforms of α1C have also been discovered in the human L-VDCC. The novel exon of the human α1C gene, exon 1a, encodes a 46-aa section at the beginning of the N terminus of α1C (the human long-NT isoform), highly homologous to the rabbit long-NT (44, 45). The previously known isoform, human short-NT, contains exon 1b at the beginning of the N terminus, which encodes a section of 16 aa (46). This isoform, used by Bourron et al. (36) and Oz et al. (37), is not up-regulated by PKC, probably because it does not contain the segment crucial for the PKC-induced enhancement of L-VDCC. The long-NT isoform of human L-VDCC, which does contain the crucial segment, is enhanced by PMA (44).

We hypothesized that it would be possible to reconstitute the enhancing effect of AngII and ACh on L-VDCC in a heterologous expression system using the long-NT isoform of α1C. Such reconstitution may greatly facilitate further studies of molecular mechanisms of L-VDCC modulation by neurotransmitters. Here, we demonstrate the reconstitution of the enhancing effect of Gq-coupled receptors on L-VDCC in Xenopus oocytes. The pharmacological characteristics of this modulation are presented. The initial segment of the N terminus is crucial for AngII- and ACh-induced enhancement of the Ca2+ channel current. The activation of Gq and the subsequent activation of PKC are clearly involved. The long human isoform of α1C is modulated in a manner similar to the long rabbit isoform, whereas the human short isoform yields currents that are only inhibited by AngII and ACh.

EXPERIMENTAL PROCEDURES

Oocyte Culture—Xenopus laevis frogs were maintained and dissected as described (47). Oocytes were injected with equal amounts (by weight; 2.5 or 1 ng) of the mRNAs of α1C or its mutants with αδδδ, with or without β2δ, with or without 1 ng of mIR or 5 ng of ATIR or ATIRMs, and incubated for 3–5 days at 20–22 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 2.5 mM sodium pyruvate, 50 µg/ml gentamycin, 5 mM HEPES, pH 7.5).

Electrophysiology—Whole cell currents were recorded using the Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA) using the two-electrode voltage clamp technique in a solution containing 40 mM Ba(OH)2, 50 mM NaOH, 2 mM KOH, and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid (48). Ca2+ currents were recorded in the same solution but with 40 mM Ca(OH)2 instead of Ba(OH)2. Stock solutions of AngII (10 mM) and ACh (1 mM) were stored in 10–20-µl aliquots at −20 °C and added to the recoding solution at a final concentration of 1 and 10 µM, respectively (except for the AngII dose-response experiment). Ba2+ currents were measured by 200-ms steps to +20 mV from a holding potential of ~80 mV, every 30 s. U73122, bis-indolylmaleimide (Bis), and staurosporine were prepared essentially as described (49, 50). In brief, U73122 was dissolved in MeSO at 20 mM and stored in 10-µl aliquots at −20 °C. Oocytes were injected with 25 nl of 600 µM U73122 and incubated in 10 µM U73122 for 30 min prior to measurements. Oocytes were injected with 50 nl of 300 µM Bis and incubated in 5 µM Bis for 2–4 h before measurement. Oocytes were incubated in 3 µM staurosporine for 2–4 h before measurement. In most experiments, all oocytes were injected with 25 nl of 50 mM BAPTA or EGTA, 30 min or 2–4 h before measurement, respectively, unless otherwise stated. All organic reagents were purchased from Sigma.

cDNA Constructs and mRNA—cDNAs of α1C, αδδδ, and β2δ were as described (51). The rabbit heart α1C mutants used here were prepared in our laboratory as described (42). cDNA of human short-NT isoform α1C was prepared (Ref. 50; GenBankTM accession number Z34815) into pGEM-HJ vector as described (44). cDNA of human long-NT isoform was constructed in our laboratory as described (44). Rat m1R cDNA is in pGEM2. Rat AT1R and AT1RMs are in pZeo (53). The RNAs were prepared using a standard procedure described previously, which ensures capping of the 5′ end of the RNA and preferential inclusion of non-capped GTP in the rest of the RNA (47).

Statistics and Data Presentation—The data are presented as mean ± S.E., n = number of cells tested. To overcome the problem of batch-to-batch variability in current amplitudes, the results were normalized as follows; in each oocyte, Ibwas was normalized to the basal amplitude (measured before application of an agonist). These normalized values were averaged across all oocytes in the same group, where two groups (e.g. control and receptor-expressing groups) were tested for statistically significant differences (p < 0.05 or better) using two-tailed unpaired t test. Comparisons of amplitudes of Ibwas at different times in the same group were done using paired t test. Comparison between several groups was done using one-way analysis of variance (ANOVA) followed by Tukey’s tests, using the SigmaStat software (SPSS Corp.).

RESULTS

Reconstitution of Neurotransmitter Modulation of L-type Ca2+ Channel in Xenopus Oocytes—We have previously demonstrated that the long-NT isoform of rabbit cardiac L-VDCC, expressed in Xenopus oocytes, is modulated by PKC activators as in cardiac and some smooth muscle cells; PMA caused an initial increase in Ba2+ current via the channels (Iba) followed by a decrease (42). In an attempt to similarly reconstitute the modulation of this channel by Gq-activating neurotransmitters (AngII, ACh), we expressed the relevant receptors (AT1R and mIR or m3R) in conjunction with the subunits of rabbit cardiac L-VDCC: α1C (the long-NT isoform; Ref. 39), αδδδ, and usually also β2δ. Xenopus oocytes were injected with the designated RNAs, and Ba2+ currents were measured using the two-electrode voltage clamp technique. The mIR was selected for practical reasons; it is known to be a Gq-coupled receptor, and it proved to be well expressed in oocytes. The m3R gave similar effects (data not shown).

The expression of mIR and AT1R was confirmed by measuring Cl− currents that develop following activation of the receptor (Fig. 1A). The appearance of this characteristic response is due to the activation of the Gq signaling cascade, which eventually leads to release of Ca2+ from intracellular stores and the consequent activation of Ca2+-dependent Cl− channels found in the oocytes (54). To avoid the development of Cl− currents while measuring Ibwas, oocytes were injected with 25 nl of 50 mM BAPTA or EGTA 1–2 h prior to current measurements (42). We did not observe any differences in the effects of AngII or ACh with either chelator; therefore, the results with BAPTA and EGTA were pooled. Ibwas was measured by step depolarizations to +20 mV from a holding potential of ~80 mV every 30 s (Fig. 1B). After allowing the current to stabilize, agonist was applied for 5 min and then washed out. Application of either ACh or AngII in oocytes that expressed the channel alone did not cause any changes in Ibwas (Fig. 1C, a). In contrast, in oocytes that expressed the channel and the receptor, both ACh and AngII caused an increase in the amplitude of Ibwas which reached a maximum after about 5 min. The enhancement of Ibwas by AngII was dose-dependent with an apparent EC50 of slightly less than 1 nM, which is similar to the known affinity range in native tissues (Fig. 1D). Following the period of increase, Ibwas declined within the next several minutes, normally below the initial (control) level (Fig. 1C, b and c). A similar decline also occurred in the constant presence of the agonist, when the latter has not been washed out after 5 min (data not shown). The reduction of Ibwas did not subside even after long periods of wash, and repetitive applications of AngII did not produce additional responses (neither increase nor decrease). The irreversibility of the decay, as well as other pa-
were applied to oocytes expressing L-VDCC composed of rabbit long-NT agonists. Modulation is only apparent in oocytes expressing the channel and a receptor.

D current measured at $t$. 

mutants of crucial for this modulation. Two different N terminus deletion PKC, the initial segment of the N terminus should also be Consequently, if the effect of ACh and AngII is mediated by activators (PMA), the initial segment of the N terminus was deleted from the N terminus ($\alpha_{1C}$, $\beta_3$, and $\beta$ subunits (a), and in addition, m1R (b) + AT1R (c)). Horizontal bars show the time of application of the corresponding agonists. Modulation is only apparent in oocytes expressing the channel and a receptor. D, AngII dose-response. Different AngII concentrations were applied to oocytes expressing L-VDCC composed of rabbit long-NT $\alpha_{1C}$, $\alpha_3$, $\beta$, and $\beta$ subunits and AT1R. The resulting responses were normalized to 1 nA AngII, which yielded the maximal response. E, summary of receptor-mediated modulation in oocytes expressing channel alone ($n = 9$), channel and m1R ($n = 45$), and channel and AT1R ($n = 27$). Black bars represent current measurement at $t = 5$ min; gray bars represent current measurements, at $t = 8$ min. The effect at 5 min in groups containing the receptors was compared with the effect in the group expressing the channels alone, by one-way ANOVA. Effects at 5 versus 8 min in each group were compared using paired t test. **, $p < 0.01$; ***, $p < 0.001$.

rameters (see below), cast doubt on its physiological relevance.

The magnitude of the enhancement of the current differed depending on the type of receptor used, possibly due to differences in the efficiency of expression. Fig. 1E summarizes the results of the experiments in which the channel was expressed in full subunit combination ($\alpha_{1C}$, $\beta_3$, $\beta_2$, $\beta_2$). Currents measured at 5 min (the peak of the increase) and at 8 min (representing the period of decline), expressed as percentage of initial IBa (measured in the same cell before application of the agonist), are shown. The increase in IBa caused by both transmitters was highly reproducible and statistically significant ($p < 0.01$). The decline phase was always present, and the decrease in IBa within a mere 3 min following the peak was also highly reproducible.

The Initial Part of the N Terminus is Crucial for Modulation by a $G_q$-coupled Receptor—In previous experiments using PKC activators (PMA), the initial segment of the N terminus was shown to be crucial for the enhancement of the current (42, 43). Consequently, if the effect of ACh and AngII is mediated by PKC, the initial segment of the N terminus should also be crucial for this modulation. Two different N terminus deletion mutants of $\alpha_{1C}$ were used in which the first 20 or 46 aa are deleted from the N terminus (D20 and D46, respectively). Oocytes that expressed the wild-type (WT) or the mutant $\alpha_{1C}$, coexpressed with $\alpha_3$ and the m1R, were subjected to a similar protocol of step depolarizations to $+20$ mV. In neither mutant was the current enhanced as a result of receptor activation by ACh. On the contrary, only a decrease was observed following activation (Fig. 2, A and B). The extent of the decrease was rather similar in the two deletion mutants tested. Thus, the first 20 aa that are crucial for up-regulation of the channel by PKC are also indispensable for the enhancement caused by the $G_q$-activating receptor, m1R (summarized in Fig. 2C). In contrast, the reduction in the current seems to be a separate effect, independent of the presence of the first 46 aa.

Transmitter-induced Modulation of Human Channel Isoforms—The up-regulation by PKC was shown to depend on the 46-aa sequence encoded in the long-NT isoform of $\alpha_{1C}$ by exon 1a (44). The alternative exon is 1h, which encodes 16 aa at the initial part of the N terminus in the short-NT isoform of $\alpha_{1C}$ (Fig. 3A). Therefore, we expected the long-NT human isoform, and not the short-NT isoform, to be regulated in a manner similar to the rabbit long-NT isoform. This prediction was fulfilled; in oocytes expressing the short-NT isoform of human $\alpha_{1C}$ along with $\alpha_3$, $\beta_2$, and m1R, there was a decrease of 18.3 ± 3% from the initial IBa at 5 min, whereas we observed a 30.8 ± 6% increase in oocytes expressing the human long-NT isoform. That is, a 49% difference in the peak current between the long and the short isoform (Fig. 3, B and C). These effects were absent in oocytes not expressing any receptor (data not shown).

Activation of $G_q$ Leads to Channel Modulation—$G_q$ normally activates phospholipase C (PLC), and the latter leads to Ca$^{2+}$ release and PKC activation. To examine whether PLC is involved in the modulation described above, we have expressed a mutated AT1 receptor in which the last five tyrosines of the C terminus were mutated to phenylalanines (AT1RM5). This mutated receptor was shown to lack $G_q$ coupling (53). Oocytes expressed the rabbit long-NT channel (in the $\alpha_{1C}$-$\alpha_3$-$\beta_2$-$\beta_3$ composition) and the wild-type receptor or the mutated one. No Ca$^{2+}$-dependent Cl$^-$ current response to AngII was observed in oocytes expressing AT1RM5 in the absence of Ca$^{2+}$ chelators (compare Fig. 4, A and B). This result confirms the lack of coupling of AT1RM5 to PLC and demonstrates that activation of this receptor does not elevate Ca$^{2+}$ in the oocytes. The
enhancement in the current via L-type Ca\(^{2+}\) channel (rabbit long-NT \(\alpha_{1C}\)) following AngII application was present only in oocytes expressing the wild-type receptor, and only a decrease was observed with AT1RM5 (Fig. 4C). Peak current, measured 5 min after AngII application, was enhanced by 18.2 \(\pm\) 4% in oocytes expressing the wild-type receptor but was decreased by 9.5 \(\pm\) 4.7% in oocytes expressing the mutated receptor. That is, a difference of 28% \((p < 0.001; \) Fig. 4D) was seen.

As an additional test for the involvement of PLC, we used the PLC inhibitor U73122. Oocytes were both injected and incubated with U73122 prior to current measurement (see “Experimental Procedures”). Treatment with U73122 of oocytes expressing the WT channel (rabbit long-NT \(\alpha_{1C}\)) and either mIR or AT1R resulted in a complete abolishment of the enhancement as compared with untreated oocytes (Fig. 5, A and B). The difference between currents with and without U73122 was 56.5% with mIR and and 34.2% with AT1R (measured after 5 min; 33.3 \(\pm\) 3.1% and 19.1 \(\pm\) 1.2% increase in untreated oocytes versus a 23.3 \(\pm\) 2.7% and 15.1 \(\pm\) 2.4% decrease in U73122-treated oocytes, respectively). In contrast, the declining phase was not attenuated by this treatment; it appeared to be augmented, possibly since the enhancement phase was inhibited. Taken together, the data described in this section demonstrate the role of \(G_{q}\) and the PLC cascade in up-regulation of the long-NT isoform of L-VDCC.

We also examined the effect of U73122 on oocytes expressing the \(\Delta N_{2-46}\) N terminus deletion mutant along with mIR. As in

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**Fig. 2.** The initial part of the N terminus is crucial for modulation by a \(G_q\)-coupled receptor. All results are from oocytes injected with BAPTA or EGTA (25 nl of 50 mM). A, a scheme of the DNA sequences corresponding to proposed alternative splice variants of the CACNA1C gene, encoding long- and short-NT isoforms of \(\alpha_{1C}\), with boxes representing exons (44). B, time course of ACh effect in oocytes expressing the channel (\(\alpha_{1C}, \alpha_{\beta}\) and \(\beta_{\alpha}\) composition) and mIR. An increase in the current is apparent in oocytes expressing the channel subunits (rabbit long-NT \(\alpha_{1C}\)) following AngII application was present only in oocytes expressing the wild-type receptor, and only a decrease was observed with AT1RM5 (n = 10, closed circles); \(\Delta N_{2-46}\) (n = 4, open circles); \(\Delta N_{2-46}\) (n = 5, closed triangles), and \(\alpha_{1C}\) and mIR. An increase in the current is only apparent in oocytes injected with the WT channel. C, summary of modulation in WT and NT deletion mutants. Black bars represent current measurements at t = 5 min; gray bars represent current measurements at t = 8 min. Statistical analysis was done only for enhancement phase. *, \(p < 0.05\) by one-way ANOVA (mutants compared with WT channel).

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**Fig. 3.** Transmitter-induced modulation of human channel isoforms. All results are from oocytes injected with BAPTA or EGTA (25 nl of 50 mM). A, a scheme of the DNA sequences corresponding to proposed alternative splice variants of the CACNA1C gene, encoding long- and short-NT isoforms of \(\alpha_{1C}\), with boxes representing exons (44). B, time course of ACh effect in oocytes expressing the channel (\(\alpha_{1C}, \alpha_{\beta}\) and \(\beta_{\alpha}\) composition) and mIR. An increase in the current is apparent in oocytes expressing the channel containing the human long-NT \(\alpha_{1C}\) isoform (closed circles; n = 6) but not in oocytes expressing the human short-NT \(\alpha_{1C}\) isoform (open circles; n = 10). C, summary of ACh-induced modulation of human long- and short-NT isoforms. Black bars represent current measurement at t = 5 min; gray bars represent current measurements at t = 8 min. ***, \(p < 0.001\).

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**Fig. 4.** Activation of \(G_q\) leads to channel modulation. A, typical chloride current in response to AngII in oocytes injected with 5 ng of RNA of AT1R versus B, lack of chloride current development in oocytes injected with 5 ng of AT1RM5. No Ca\(^{2+}\) chelators have been injected into oocytes in the recordings of A and B. C, time course of AngII effect in oocytes expressing the channel subunits (rabbit long-NT \(\alpha_{1C}, \alpha_{\beta}\), \(\beta_{\alpha}\)) and AT1R (n = 7) or AT1RM5 (n = 8). The increase is absent in oocytes expressing AT1RM5. D, summary of AngII-induced modulation via AT1R or AT1RM5. I\(_{\text{Ba}}\) was measured in oocytes injected with BAPTA or EGTA (25 nl of 50 mM). Black bars represent current measurement at t = 5 min; gray bars represent current measurements at t = 8 min. Statistical analysis was done only for the enhancement phase. ***, \(p < 0.001\) by t test between AT1R and AT1RM5 groups.
PKC Is Involved in Enhancement of the Current—To substantiate the involvement of PKC in the modulation via the receptors, we used PKC inhibitors. bis-indolylmaleimide is a potent, selective inhibitor of PKC. It inhibits PKC by interacting with the catalytic subunit, thus competing with ATP (56, 57). Bis completely inhibited the enhancement of the current caused by either AngII via AT1R or ACh via m1R but did not affect the declining phase (Fig. 5A). Another PKC inhibitor tested, chelerythrin, which inhibits only Ca2+-dependent PKCs, did not affect the m1R modulation (data not shown).

Effects of Ca2+ Chelators on AngII Modulation of the Short Human Isoform of α1c—Oz et al. (37) reported that AngII-induced inhibition of L-VDCC in Xenopus oocytes expressing the short human isofrom of α1c was fully eliminated by injecting BAPTA into the oocytes prior to measuring the current. This result, along with additional observations, led Oz et al. (37) to suggest that the AngII-induced decrease in I\(_{\text{Ba}}\) was mediated via the Gq-Ca2+ release pathway. This seems to be incompatible with our suggestion that Gq is not involved in this effect, at least regarding the long-NT isofrom. One possibility is that the mechanisms of the decrease in I\(_{\text{Ba}}\) are not identical in long- and short-NT isofroms. Although at present we cannot rule out this notion, it seems unlikely because the kinetics and extent of this effect are similar in both isofroms, and high doses of Ca2+-chelators eliminated this modulation in both isofroms (see below). Another possibility is a difference in experimental procedures; Oz et al. (37) injected four times the amount of BAPTA that we have used here. To address this possibility, we expressed the short-human isoform with AT1R and performed the experiment under three conditions: no chelator injected to the oocytes prior to current measurements; with our standard concentration of EGTA or BAPTA (25 nl of 50 mM); and with a high BAPTA concentration (50 nl of 100 mM). The inhibition was strongest without any chelator (Fig. 6A); however, the measurement of I\(_{\text{Ba}}\) was less reliable than in other groups due to the presence of Cl- currents. The AngII-induced inhibition of I\(_{\text{Ba}}\) was significantly reduced in oocytes with low doses of both chelators and abolished in oocytes injected with the high BAPTA concentration (Fig. 6A). The Ca2+-dependent nature of the AngII-induced inhibition was further confirmed by examining the effects of AngII in oocytes injected with the rabbit long-NT isoform of the channel and AT1R, when Ca2+, rather than Ba2+, was used as the charge carrier. In this case as well, the increase in the current in response to agonist was followed by a decrease that was substantially stronger than with Ba2+ (Fig. 6B; compare with Fig. 1, C and E). These results confirm those of Oz et al. (37) and imply a substantial Ca2+ dependence of the inhibitory effect. However, they still do not rule out the possibility that this phenomenon is not mediated by the Gq-α1c-dependent increase in Ca2+ but by a different mechanism that requires the presence of basol levels of Ca2+ (and is therefore eliminated by Ca2+ chelation). Therefore, we expressed the short-human isoform with the mutated receptor AT1RM5, which lacks Gq coupling and does not elevate Ca2+, and performed the experiment under two conditions: no chelator injected and high BAPTA concentration injected. The inhibition of the current was abolished in oocytes injected with BAPTA (Fig. 6C). To further examine this conundrum, we expressed the long-rabbit isoform of α1c with α2/β3, and AT1RM5 and injected the oocytes with the standard concentration of Ca2+-chelators used here (25 nl of 50 mM), no chelator, or the high

The decrease was even stronger in U73122-treated oocytes: 21.4 ± 3.6% in untreated oocytes, 39.0 ± 2.5% in U73122-treated oocytes (measured after 5 min; p < 0.01). These results imply that some enhancing effect of PLC was still present in the ΔN\(_{2-46}\) mutant, despite the apparent absence of the enhancement phase. Alternatively, U73122 may enhance the (unknown) mechanism by which ACh inhibits L-VDCC.

the previous experiments, in untreated ΔN\(_{2-46}\) oocytes, we observed only a decrease and no enhancement phase (Fig. 5C). The decrease was even stronger in U73122-treated oocytes: 21.4 ± 3.6% in untreated oocytes, 39.0 ± 2.5% in U73122-treated oocytes (measured after 5 min; p < 0.01). These results imply that some enhancing effect of PLC was still present in the ΔN\(_{2-46}\) mutant, despite the apparent absence of the enhancement phase. Alternatively, U73122 may enhance the (unknown) mechanism by which ACh inhibits L-VDCC.

PKC Is Involved in Enhancement of the Current—To substantiate the involvement of PKC in the modulation via the receptors, we used PKC inhibitors. bis-indolylmaleimide is a potent, selective inhibitor of PKC. It inhibits PKC by interacting with the catalytic subunit, thus competing with ATP (56, 57). Bis completely inhibited the enhancement of the current caused by either AngII via AT1R or ACh via m1R but did not affect the declining phase (Fig. 5A). Another PKC inhibitor tested, chelerythrin, which inhibits only Ca2+-dependent PKCs, did not affect the m1R modulation (data not shown).

Effects of Ca2+ Chelators on AngII Modulation of the Short Human Isoform of α1c—Oz et al. (37) reported that AngII-induced inhibition of L-VDCC in Xenopus oocytes expressing the short human isofrom of α1c was fully eliminated by injecting BAPTA into the oocytes prior to measuring the current. This result, along with additional observations, led Oz et al. (37) to suggest that the AngII-induced decrease in I\(_{\text{Ba}}\) was mediated via the Gq-Ca2+ release pathway. This seems to be incompatible with our suggestion that Gq is not involved in this effect, at least regarding the long-NT isofrom. One possibility is that the mechanisms of the decrease in I\(_{\text{Ba}}\) are not identical in long- and short-NT isofroms. Although at present we cannot rule out this notion, it seems unlikely because the kinetics and extent of this effect are similar in both isofroms, and high doses of Ca2+-chelators eliminated this modulation in both isofroms (see below). Another possibility is a difference in experimental procedures; Oz et al. (37) injected four times the amount of BAPTA that we have used here. To address this possibility, we expressed the short-human isoform with AT1R and performed the experiment under three conditions: no chelator injected to the oocytes prior to current measurements; with our standard concentration of EGTA or BAPTA (25 nl of 50 mM); and with a high BAPTA concentration (50 nl of 100 mM). The inhibition was strongest without any chelator (Fig. 6A); however, the measurement of I\(_{\text{Ba}}\) was less reliable than in other groups due to the presence of Cl- currents. The AngII-induced inhibition of I\(_{\text{Ba}}\) was significantly reduced in oocytes with low doses of both chelators and abolished in oocytes injected with the high BAPTA concentration (Fig. 6A). The Ca2+-dependent nature of the AngII-induced inhibition was further confirmed by examining the effects of AngII in oocytes injected with the rabbit long-NT isoform of the channel and AT1R, when Ca2+, rather than Ba2+, was used as the charge carrier. In this case as well, the increase in the current in response to agonist was followed by a decrease that was substantially stronger than with Ba2+ (Fig. 6B; compare with Fig. 1, C and E). These results confirm those of Oz et al. (37) and imply a substantial Ca2+ dependence of the inhibitory effect. However, they still do not rule out the possibility that this phenomenon is not mediated by the Gq-α1c-dependent increase in Ca2+ but by a different mechanism that requires the presence of basol levels of Ca2+ (and is therefore eliminated by Ca2+ chelation). Therefore, we expressed the short-human isoform with the mutated receptor AT1RM5, which lacks Gq coupling and does not elevate Ca2+, and performed the experiment under two conditions: no chelator injected and high BAPTA concentration injected. The inhibition of the current was abolished in oocytes injected with BAPTA (Fig. 6C). To further examine this conundrum, we expressed the long-rabbit isoform of α1c with α2/β3, and AT1RM5 and injected the oocytes with the standard concentration of Ca2+-chelators used here (25 nl of 50 mM), no chelator, or the high
BAPTA concentration. The decrease in the current was apparent only in oocytes injected with the low chelator concentration or no chelator, but it was smaller than the decrease observed with the wild type AT1R under the conditions of U73122-inhibited Gq:20% (n = 17) versus 35 ± 2.6% (n = 6), respectively, at 8 min in low chelator (p < 0.001). The decrease caused by activation of AT1RM5 in the long-NT isoform current was eliminated with the higher concentration of BAPTA (Fig. 6D).

DISCUSSION

Our results demonstrate modulation of L-type Ca\(^{2+}\) channel by a neurotransmitter (ACh) and a hormone (AngII) known to activate Gq and enhance L-type Ca\(^{2+}\) currents in heart and/or smooth muscle. AT1R or m1R were expressed in Xenopus oocytes in combination with the rabbit long-NT \(\alpha_{1c}\), \(\beta_{2a}\), and \(\alpha_{f/b}\). Application of the agonists resulted in an enhancement of the current that developed over 5 min, subsequently subsided, and was followed by a decrease below baseline values. The up-regulation of the Ca\(^{2+}\) channel current, which is in the focus of this study, involves activation of Gq, as well as other components downstream: PLC and PKC. The initial part of the N terminus, known to be important for PKC regulation, is shown to be crucial for this modulation. Finally, human long isoform of \(\alpha_{1c}\) behaves similarly to the long rabbit isoform. The enhancement of L-VDCC currents by AngII and ACh, or any Gq-activating neurotransmitter, has been reconstituted in a heterologous expression system for the first time, providing a powerful tool for further studies of this modulation.

Following reconstitution of the up-regulation, involvement of Gq was examined. Inhibition of PLC, located downstream of Gq activation, abolished the enhancement of the current. Furthermore, expression of a mutant AT1 receptor, which lacks Gq coupling, did not yield a current increase either. This clearly demonstrates the involvement of Gq in the modulation.

Several lines of evidence suggest that the enhancement of Ca\(^{2+}\) channel current by the transmitters studied was mediated by PKC. Inhibition of PKC reduced the extent of up-regulation of the current. Two different PKC inhibitors that we used, Bis and staurosporine, have both significantly attenuated the enhancing effect of AngII and ACh. The assertion that the ACh- and AngII-induced increase in Ca\(^{2+}\) channel current is mediated by PKC is also supported by the absence of this modulation in mutants lacking the initial 20 aa of the NT, known to be crucial for the PKC effect, and in the short-NT isoform; these constructs have been shown previously to lack...
the enhancing phase of modulation by a PKC-activating phospholipid ester, PMA (43, 44).

The exact mechanism by which PKC enhances the current is still not clear. PKC enhances L-, N-, and P/Q-type currents; N and P/Q channels are phosphorylated by PKC at different sites in the L1 loop, and this attenuates the inhibition of the channel by Gβγ (60). In α1C, the mechanism is different. Neither the crucial initial 20 aa of NT nor the L1 loop are directly phosphorylated by PKC (42, 43). Thus modulation by PKC may be direct or indirect. For instance, activated PKC may phosphorylate a segment of the channel that interacts with the NT. Alternatively, PKC may modify the transport of the channel to the plasma membrane, thus leading to an increase in the current.

The present study resolves the controversy regarding the modulation of mammalian, including human, L-VDCCC by Gq and PKC-activating neurotransmitters. Despite the widely recognized enhancing action of AngII (via AT1) in cardiac and many smooth muscle cells and of ACh (via m3) in smooth muscle cells, this effect could not be reproduced in heterologous expression systems. Our results suggest that the problem may lie in the different modulation of isoforms of α1C by these transmitters. Only the long-NT isoform of α1C, long known in rabbit and rat but only recently discovered in humans, is up-regulated by PKC in the oocytes (44); therefore, it is not surprising that only a decrease in the current following agonist application is observed with the short-NT isoform used in the previous studies of PKC and AngII modulation of human L-type channels expressed in Xenopus oocytes (36, 37). Another obstacle to study PKC modulation of L-type Ca2+ channels may be the use of heterologous expression systems apparently lacking one of the essential components required for the modulation. McHugh et al. (61) reported that activation of PKC only decreased L-type Ca2+ current via channels based on long-NT rabbit α1C expressed in HEK cells. They suggested that HEK cells might be lacking the appropriate PKC isoform. Although at present we do not know which PKC isoform is involved in the mediation of AngII- and ACh-induced enhancement in Xenopus oocytes, it is probably a Ca2+-independent one because the increase is not blocked by chelation of intracellular Ca2+ and by chelerythrin, an inhibitor of "classical," Ca2+-dependent PKC isoforms α, β, or γ (62). The Xenopus oocyte, which faithfully reproduces the well described enhancing effect of the ACh and AngII, may be the system of choice for further molecular studies of this type of regulation of the L-type channels.

The inhibitory effect of AngII and ACh was not in the focus of previous studies of PKC and AngII modulation of human L-type channels by neurokinin 1 receptor AT1RM5 and because the PLC inhibitor, U73122, did not affect it. The potency of AT1RM5 in decreasing IBa in oocytes is also apparent upon application of PMA, which activates PKC directly (42, 63). It is possible to coalesce these results by assuming that the proposed unknown pathway leads to the activation of a PKC isoform with low sensitivity to Bis. This is supported by the observation that staurosporine, a more potent PKC inhibitor, blocked the decrease. Also, another protein kinase may be involved since staurosporine is a broad serine/threonine kinase inhibitor (58, 59), but this does notexplain the inhibitory effect of PMA. Alternatively, the inhibition by PMA and by AngII (ACh) may be mediated by different mechanisms.

The decrease in Ba2+ current via rabbit long-NT channels reported by McHugh et al. (61) in HEK cells was eliminated by mutating two threonines at positions 27 and 31, and the authors speculated that these two residues are phosphorylated by PKC and that this phosphorylation underlies the observed decrease (61). In contrast, in oocytes, the PMA-, AngII-, and ACh-induced decrease remains intact in constructs lacking the first 46 aa that include the two serines. It appears that PMA-induced decreases in IBa in HEK cells and in oocytes are mediated by different molecular mechanisms.

The Gq-independent inhibition of the current via the short-NT human α1C isoform caused by AngII was Ca2+-dependent, but it was not Ca2+-mediated. We speculate that basal levels of intracellular Ca2+ are crucial for this portion of the decrease in the current. One possibility is that the inhibition relies on proper folding of the proximal part of cytosolic C terminus of α1C, which requires the presence of basal Ca2+ levels (55). Further studies are required to understand the mechanism of the AngII- and ACh-induced inhibition and to establish whether it is apparent in cardiac and smooth muscle tissues.

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