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Modulation of Cardiac Ca^{2+} Channel by G_q-activating Neurotransmitters Reconstituted in *Xenopus* Oocytes*

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L-type dihydropyridine-sensitive voltage dependent Ca^{2+} channels (L-VDCCs; α_{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 α_{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 α_{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)[1] 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 adenylyl 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_{Ba} was inhibited. Oz *et al.* (37) reported a decrease in Ca^{2+} current in oocytes expressing α_{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-

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[1] The abbreviations used are: L-VDCC, L-type voltage dependent Ca^{2+} channels; aa, amino acid; ACh, acetylcholine; AngII, angiotensin II; AT1R, angiotensin II receptor type 1; Bis, bis-indolylmaleimide; m1R and m3R, muscarinic receptors 1 and 3; NT, N-terminus; PKC, protein kinase C; PLC, phospholipase C; WT, wild-type; PMA, 4β-phorbol-12-myristate 13-acetate; ANOVA, analysis of variance; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid.

hancement may be related to the use of certain isoforms of α_{1C} that are not modulated by PKC. In the rat and rabbit, two N-terminal (NT) isoforms of α_{1C} ($Ca_v2.1$) are known, which probably represent variable splicing products of the same gene (38). These splice variants encode long- and short-NT α_{1C} 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 α_{1C} is ~154 aa in the long-NT α_{1C}). 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 α_{1C} have also been discovered in the human L-VDCC. The novel exon of the human α_{1C} gene, exon 1a, encodes a 46-aa section at the beginning of the N terminus of α_{1C} (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 Bouron *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 α_{1C}. 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 G_q-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 Ca^{2+} channel current. The activation of G_q, and the subsequent activation of PKC, are clearly involved. The long human isoform of α_{1C} 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 α_{1C} or its mutants with α_2/δ, with or without β_{2A}, with or without 1 ng of m1R or 5 ng of AT1R or AT1RM5, and incubated for 3–5 days at 20–22 °C in NDE96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 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). Ca^{2+} 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 M) 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). Ba^{2+} 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 Me_2SO 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 α_{1C}, α_2/δ, and β_{2A} were as described (51). The rabbit heart α_{1C} mutants used here were prepared in our laboratory as described (42). cDNA of human short-NT isoform $\alpha_{1C,77}$ (Ref. 52; GenBank™ accession number Z34815) was subcloned 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 AT1RM5 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, I_{Ba} was normalized to the basal amplitude (measured before application of an agonist). These normalized values were averaged across all oocyte batches tested. Comparisons between 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 I_{Ba} 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 Ca^{2+} 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 Ba^{2+} current via the channels (I_{Ba}) followed by a decrease (42). In an attempt to similarly reconstitute the modulation of this channel by G_q-activating neurotransmitters (AngII, ACh), we expressed the relevant receptors (AT1R and m1R or m3R) in conjunction with the subunits of rabbit cardiac L-VDCC: α_{1C} (the long-NT isoform; Ref. 39), $\alpha_2\delta$, and usually also β_{2A}. *Xenopus* oocytes were injected with the designated RNAs, and Ba^{2+} currents were measured using the two-electrode voltage clamp technique. The m1R was selected for practical reasons; it is known to be a G_q-coupled receptor, and it proved to be well expressed in oocytes. The m3R gave similar effects (data not shown).

The expression of m1R and AT1R was confirmed by measuring Cl^- currents that develop following activation of the receptor (Fig. 1*A*). The appearance of this characteristic response is due to the activation of the G_q signaling cascade, which eventually leads to release of Ca^{2+} from intracellular stores and the consequent activation of Ca^{2+}-dependent Cl^- channels found in the oocytes (54). To avoid the development of Cl^- currents while measuring I_{Ba}, 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. I_{Ba} was measured by step depolarizations to +20 mV from a holding potential of −80 mV every 30 s (Fig. 1*B*). 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 I_{Ba} (Fig. 1*C*, *a*). In contrast, in oocytes that expressed the channel and the receptor, both ACh and AngII caused an increase in the amplitude of I_{Ba}, which reached a maximum after about 5 min. The enhancement of I_{Ba} by AngII was dose-dependent with an apparent EC_{50} of slightly less than 1 nM, which is similar to the known affinity range in native tissues (Fig. 1*D*). Following the period of increase, I_{Ba} declined within the next several minutes, normally below the initial (control) level (Fig. 1*C*, *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 I_{Ba} 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-

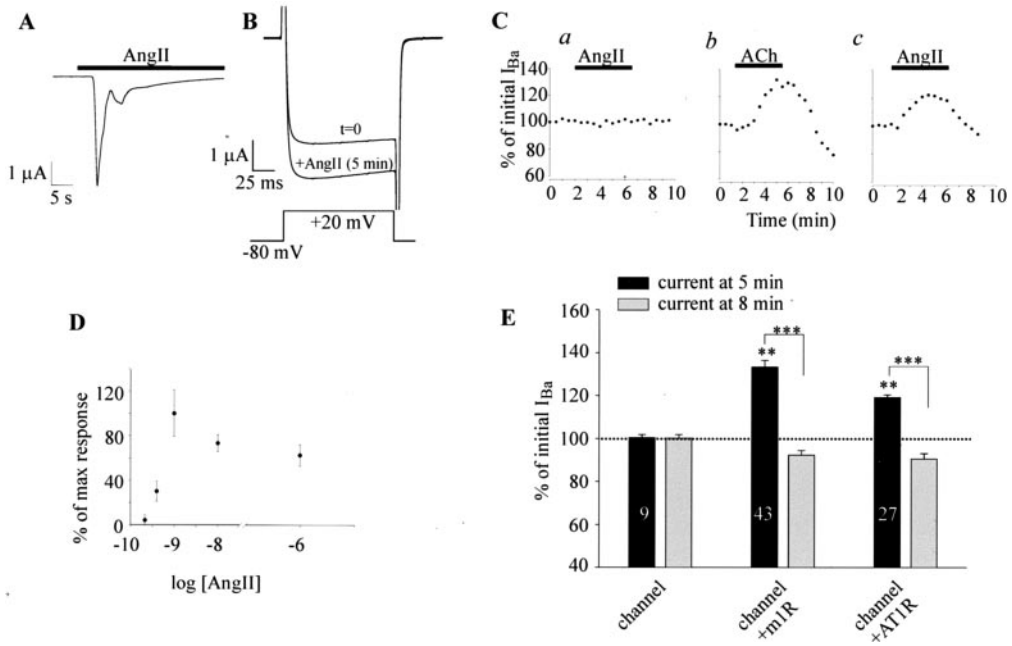

FIG. 1. **Reconstitution of neurotransmitter modulation of L-type Ca^{2+} channel in *Xenopus* oocytes.** *A*, typical chloride current in response to agonist (AngII) in oocytes expressing AT1R. Currents were measured in a solution containing 40 mM Ba^{2+}, in the absence of Ca^{2+} chelators. *B*, *lower trace*, experimental protocol to measure I_{Ba}: a step depolarization to +20 mV from a holding potential of −80 mV. *Upper traces*, currents measured at $t = 0$ min, and at $t = 5$ min after the addition of ACh, are shown. To measure I_{Ba}, oocytes were injected with standard BAPTA or EGTA concentrations (25 nl of 50 mM). *C*, typical time course of changes in I_{Ba} in representative oocytes expressing L-VDCC composed of rabbit long-NT α_{1C}, α_2/δ, and β subunits (*a*), and in addition, m1R (*b*) r 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 α_{1C}, α_2/δ, and β subunits and AT1R. The resulting responses were normalized to 1 nM AngII, which yielded the maximal response. *E*, summary of receptor-mediated modulation in oocytes expressing channel alone ($n = 9$), channel and m1R ($n = 43$), 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. 1*E* summarizes the results of the experiments in which the channel was expressed in full subunit combination ($\alpha_{1C}\alpha_2/\delta\beta_{2A}$). Currents measured at 5 min (the peak of the increase) and at 8 min (representing the period of decline), expressed as percentage of initial I_{Ba} (measured in the same cell before application of the agonist), are shown. The increase in I_{Ba} caused by both transmitters was highly reproducible and statistically significant ($p < 0.01$). The decline phase was always present, and the decrease in I_{Ba} 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 α_{1C} were used in which the first 20 or 46 aa are deleted from the N terminus (ΔN_{2-20} and DN_{2-46}, respectively). Oocytes that expressed the wild-type (WT) or the mutant α_{1C}, coexpressed with α_2/δ 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. 2*C*). 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 α_{1C} by exon 1a (44). The alternative exon is 1b, which encodes 16 aa at the initial part of the N terminus in the short-NT isoform of α_{1C} (Fig. 3*A*). 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 α_{1C} along with $\alpha_2\delta$, β_{2A}, and m1R, there was a decrease of 18.3 ± 3% from the initial I_{Ba} 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 $\alpha_{1C}\alpha_2/\delta\beta_{2A}$ 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

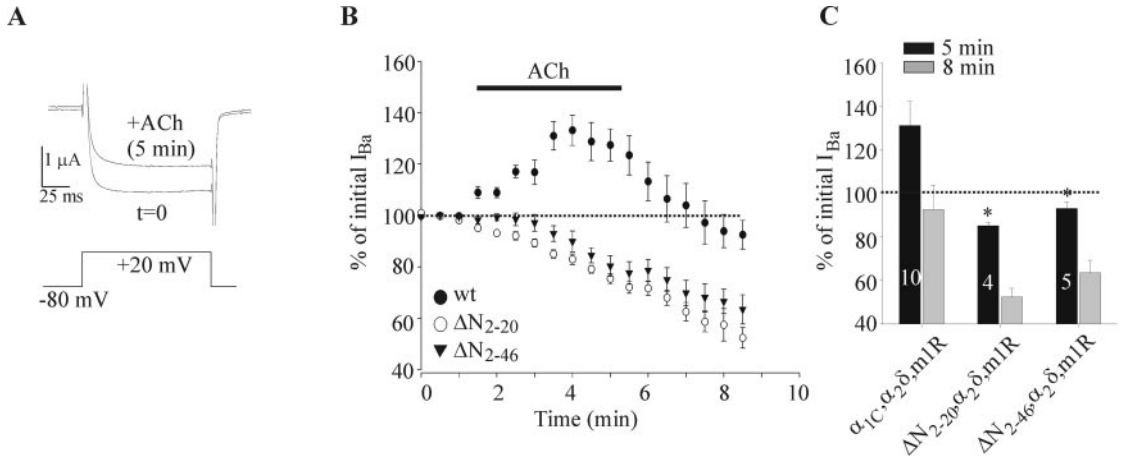

FIG. 2. **The initial part of the N terminus is crucial for modulation by a G_q-coupled receptor.** All results are from oocytes injected with BAPTA or EGTA (25 nl of 50 mM). *A*, typical I_{Ba} in oocytes expressing the α_{1C} mutant ΔN_{2-46}, α_2/δ, β_{2A}, and m1R, measured at $t = 0$ min and $t = 5$ min. *B*, time course of ACh effects in oocytes expressing rabbit long WT α_{1C} ($n = 10$, *closed circles*); ΔN_{2-20} ($n = 4$, *open circles*); ΔN_{2-46} ($n = 5$, *closed triangles*), and $\alpha_2/\delta$and m1R. 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).

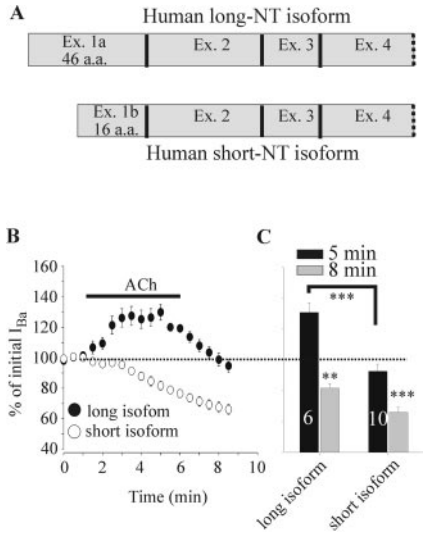

FIG. 3. **Transmitter-induced modulation of human channel isoforms.** All results are from oocytes injected with BAPTA or EGTA (25 nl of 50 mM). *A*, a scheme of the DNA sequences corresponding to proposed alternative splice variants of the *CACNA1C* gene, encoding long- and short-NT isoforms of α_{1C}, with *boxes* representing exons (44). *B*, time course of ACh effect in oocytes expressing the channel ($\alpha_{1C}\alpha_2/\delta\beta_{2A}$ composition) and m1R. An increase in the current is apparent in oocytes expressing the channel containing the human long-NT α_{1C} isoform (*closed circles*; $n = 6$) but not in oocytes expressing the human short-NT α_{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. **, $p < 0.01$; ***, $p < 0.001$.

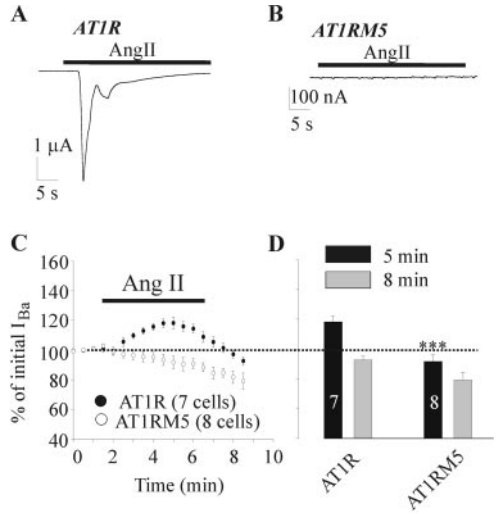

FIG. 4. **Activation of G_q leads to channel modulation.** *A*, typical chloride current in response to AngII in oocytes injected with 5 ng of RNA of AT1R *versus* *B*, lack of chloride current development in oocytes injected with 5 ng of AT1RM5. No Ca^{2+} chelators have been injected into oocytes in the recordings of *A* and *B*. *C*, time course of AngII effect in oocytes expressing the channel subunits (rabbit long-NT α_{1C}, α_2/δ, β_{2A}) 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_{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.

enhancement in the current via L-type Ca^{2+} channel (rabbit long-NT α_{1C}) following AngII application was present only in oocytes expressing the wild-type receptor, and only a decrease was observed with AT1RM5 (Fig. 4*C*). Peak current, measured 5 min after AngII application, was enhanced by 18.2 ± 4% in oocytes expressing the wild-type receptor but was decreased by 9.5 ± 4.7% in oocytes expressing the mutated receptor. That is, a difference of 28% ($p < 0.001$; Fig. 4*D*) 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 α_{1C}) and either m1R 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 m1R and and 34.2% with AT1R (measured after 5 min; 33.3 ± 3.1% and 19.1 ± 1.2% increase in untreated oocytes *versus* a 23.3 ± 2.7% and 15.1 ± 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 ΔN_{2-46} N terminus deletion mutant along with m1R. As in

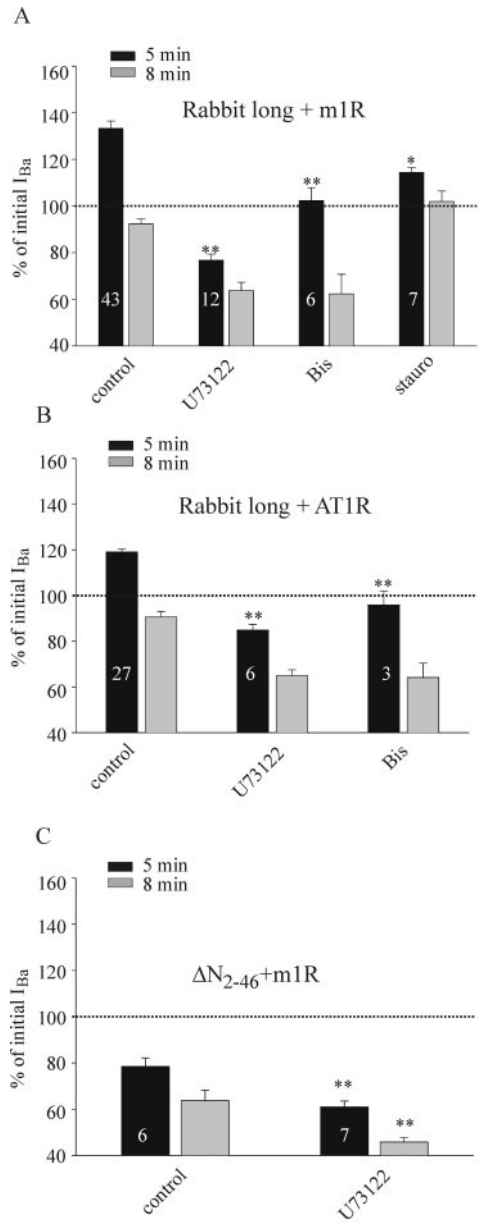

FIG. 5. **PKC and PLC are involved in the modulation.** *Black bars* represent current measurement at $t = 5$ min (after the corresponding agonists were applied); *gray bars* represent current measurements at $t = 8$ min. All results are from oocytes injected with BAPTA or EGTA (25 nl of 50 mM). In *A* and *B*, the effects of agonists at 5 min have been compared for statistical significance with the control group (no treatment; *leftmost black bar*) by one-way ANOVA. *A*, summary of effects of U73122, Bis, and staurosporine (*stauro*) in oocytes expressing rabbit long-NT α_{1C} (WT), $\alpha_2\delta$, β, and m1R. *B*, summary of effects of U73122 and Bis in oocytes expressing rabbit long-NT α_{1C} (WT), $\alpha_2\delta$, β_{2A}, and AT1R. *C*, summary of effects of U73122 in oocytes expressing the ΔN_{2-46} α_{1C} mutant, $\alpha_2\delta$, β, and m1R. Untreated oocytes ($n = 6$) were compared with U73122-treated oocytes ($n = 7$) by t test. Statistical analysis was done both for the enhancement phase and for the decline phase. *, $p < 0.05$; **, $p < 0.01$.

the previous experiments, in untreated ΔN_{2-46} oocytes, we observed only a decrease and no enhancement phase (Fig. 5*C*). 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. 5, *A* and *B*). Treatment with staurosporine, a protein kinase inhibitor with a broad spectrum of action that shows only partial specificity toward PKC (58, 59), partly inhibited the increase in the current evoked by ACh activation of m1R. Interestingly, it also inhibited most of the decrease in the current (Fig. 5*A*). Another PKC inhibitor tested, chelerythrin, which inhibits only Ca^{2+}-dependent PKCs, did not affect the m1R modulation (data not shown).

Effects of Ca^{2+} Chelators on AngII Modulation of the Short Human Isoform of α_{1C}—Oz *et al.* (37) reported that AngII-induced inhibition of L-VDCC in *Xenopus* oocytes expressing the short human isoform of α_{1C} 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_{Ba} was mediated via the G_q-Ca^{2+} release pathway. This seems to be incompatible with our suggestion that G_q is not involved in this effect, at least regarding the long-NT isoform. One possibility is that the mechanisms of the decrease in I_{Ba} are not identical in long- and short-NT isoforms. Although at present we cannot rule out this notion, it seems unlikely because the kinetics and extent of this effect are similar in both isoforms, and high doses of Ca^{2+} chelators eliminated this modulation in both isoforms (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. 6*A*); however, the measurement of I_{Ba} was less reliable than in other groups due to the presence of Cl^- currents. The AngII-induced inhibition of I_{Ba} was significantly reduced in oocytes with low doses of both chelators and abolished in oocytes injected with the high BAPTA concentration (Fig. 6*A*). The Ca^{2+}-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 Ca^{2+}, rather than Ba^{2+}, 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 Ba^{2+} (Fig. 6*B*; compare with Fig. 1, *C* and *E*). These results confirm those of Oz *et al.* (37) and imply a substantial Ca^{2+} dependence of the inhibitory effect. However, they still do not rule out the possibility that this phenomenon is not mediated by the $G\alpha_q$-dependent increase in Ca^{2+} but by a different mechanism that requires the presence of basal levels of Ca^{2+} (and is therefore eliminated by Ca^{2+} chelation). Therefore, we expressed the short-human isoform with the mutated receptor AT1RM5, which lacks G_q coupling and does not elevate Ca^{2+}, 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. 6*C*). To further examine this conundrum, we expressed the long-rabbit isoform of α_{1C} with α_2/δ, β_{2A}, and AT1RM5 and injected the oocytes with the standard concentration of Ca^{2+} chelators used here (25 nl of 50 mM), no chelator, or the high

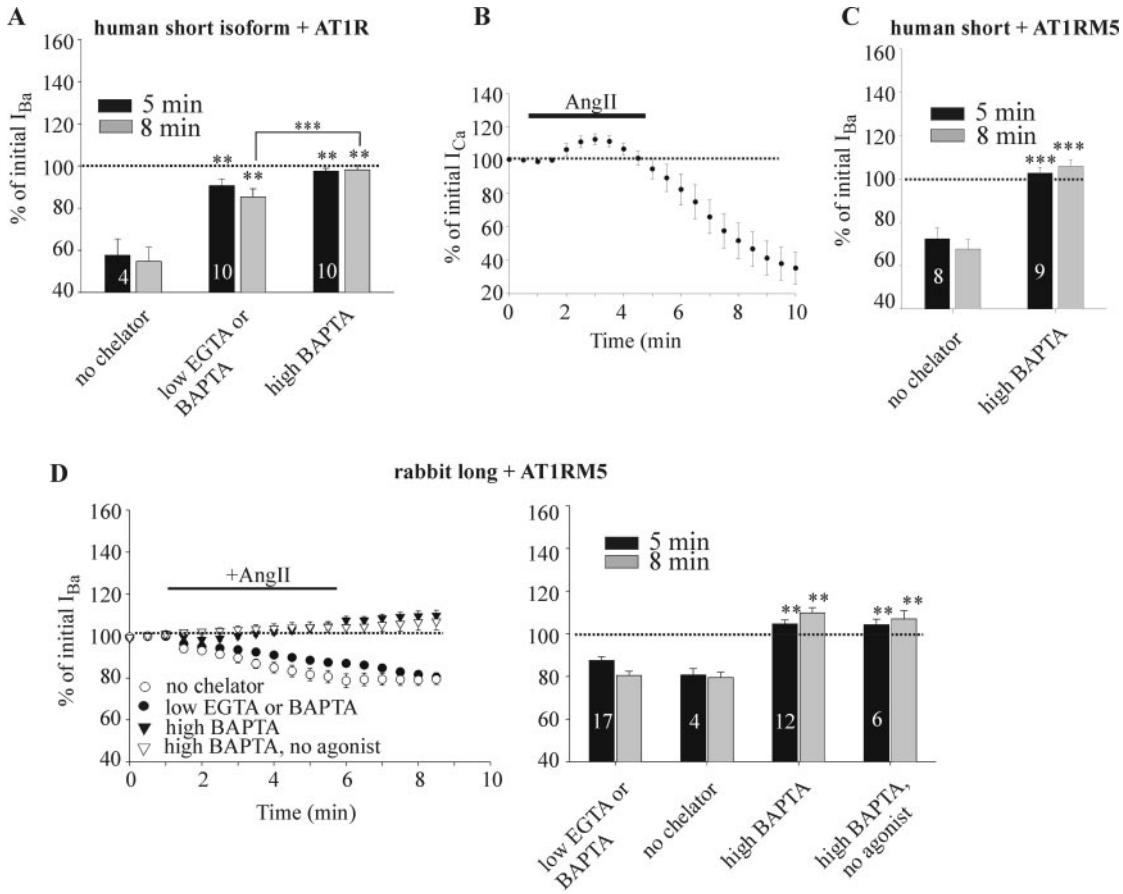

FIG. 6. **Effects of Ca^{2+} chelators on AngII modulation.** Oocytes expressed the indicated isoform of α_{1C}, α_2/δ, β_{2A}, and WT or mutated AT1R. The various oocyte groups shown were either without Ca^{2+} chelators or injected with standard "low" BAPTA or EGTA concentrations (25 nl of 50 mM) or injected with a high BAPTA concentration (50 nl of 100 mM). *Black bars* represent current measurements at $t = 5$ min (after AngII application); *gray bars* represent current measurements at $t = 8$ min. *A*, summary of experiments conducted in oocytes expressing human short-NT α_{1C} isoform and AT1R. Low BAPTA or EGTA ($n = 10$) weakened the modulation, whereas high BAPTA ($n = 10$) abolishes it, as compared with no chelation ($n = 4$). *B*, time course changes in I_{Ca} in oocytes expressing L-VDCC composed of rabbit long-NT α_{1C}, α_2/δ, and β subunits and AT1R. The *horizontal bar* shows the time of application of AngII. *C*, summary of experiments in oocytes expressing human short-NT α_{1C} isoform and AT1RM5. High BAPTA ($n = 9$) abolishes the modulation observed in oocytes without a chelator ($n = 8$). *D*, *left*, time course of experiments conducted with oocytes expressing rabbit long-NT α_{1C} isoform and AT1RM5, with and without AngII, and *D*, *right*, summary of experiments conducted with rabbit long-NT α_{1C} isoform and AT1RM5. High BAPTA ($n = 12$) abolishes the modulation as compared with oocytes without chelation ($n = 4$) or oocytes with low BAPTA/EGTA ($n = 17$). The modulation does not take place when no agonist is applied ($n = 6$). **, $p < 0.01$; ***, $p < 0.001$.

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 G_q: 20 ± 2% ($n = 17$) *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. 6*D*).

DISCUSSION

Our results demonstrate modulation of L-type Ca^{2+} channel by a neurotransmitter (ACh) and a hormone (AngII) known to activate G_q 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 α_{1C}, β_{2A}, and α_2/δ. 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 G_q, 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 α_{1C} behaves similarly to the long rabbit isoform. The enhancement of L-VDCC currents by AngII and ACh, or any G_q-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 G_q was examined. Inhibition of PLC, located downstream of G_q activation, abolished the enhancement of the current. Furthermore, expression of a mutant AT1 receptor, which lacks G_q coupling, did not yield a current increase either. This clearly demonstrates the involvement of G_q 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 phorbol 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 G$\beta\gamma$ (60). In α_{1C}, 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-VDCC by G_q- 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 α_{1C} by these transmitters. Only the long-NT isoform of α_{1C}, long known in rabbit and rat but only recently discovered in humans, is upregulated 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 Ca^{2+} 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 Ca^{2+} current via channels based on long-NT rabbit α_{1C} expressed in HEK cells. They suggested that HEK cells might be lacking the appropriate PKC isozyme. Although at present we do not know which PKC isozyme is involved in the mediation of AngII- and ACh-induced enhancement in *Xenopus* oocytes, it is probably a Ca^{2+}-independent one because the increase is not blocked by chelation of intracellular Ca^{2+} and by chelerythrin, an inhibitor of "classical," Ca^{2+}-dependent PKC isozymes 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 this study, as it is not clear whether it is physiologically relevant. No such inhibition is normally observed with G_q-activating receptors in cardiac and smooth muscle cells. The molecular mechanism of this effect is puzzling; it is clearly different from that of the enhancement. The decrease in I_{Ba} was G_q- and PLC-independent because it was induced by the G_q-uncoupled AngII receptor AT1RM5 and because the PLC inhibitor, U73122, did not affect it. The potency of AT1RM5 in decreasing I_{Ba} in oocytes appeared lower than that of the wild type receptor, but otherwise, the effects were similar. Notably, AT1RM5 retains its ability to activate an undefined G_q-independent signaling pathway(s), resulting in tyrosine-dependent phosphorylation of STAT1 (53). The AngII-induced inhibition may proceed via such a pathway. Surprisingly, current reduction 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 isozyme 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 not explain the inhibitory effect of PMA. Alternatively, the inhibition by PMA and by AngII (ACh) may be mediated by different mechanisms.

The decrease in Ba^{2+} 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 I_{Ba} in HEK cells and in oocytes are mediated by different molecular mechanisms.

The G_q-independent inhibition of the current via the short-NT human α_{1C} isoform caused by AngII was Ca^{2+}-dependent, but it was not Ca^{2+}-mediated. We speculate that basal levels of intracellular Ca^{2+} 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 α_{1C}, which requires the presence of basal Ca^{2+} 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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REFERENCES

1. Reuter, H. (1983) *Nature* **301,** 569–574
2. Hughes, A. D. (1995) *J. Vasc. Res.* **32,** 353–370
3. Xiong, Z., and Sperelakis, N. (1995) *J. Mol. Cell. Cardiol.* **27,** 75–91
4. Trautwein, W., and Hescheler, J. (1990) *Annu. Rev. Physiol.* **52,** 257–274
5. Berk, B. C., and Corson, M. A. (1997) *Circ. Res.* **80,** 607–616
6. Beech, D. J. (1997) *Pharmacol. Ther.* **73,** 91–119
7. Jackson, E. K., and Garrison, J. C. (1996) in *Goodman & Gilman's The Pharmacological Basis of Therapeutics* (Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., and Gilman, A. G., eds), pp. 733–758, McGraw-Hill, New York
8. Eglen, R. M., Reddy, H., Watson, N., and Challiss, R. A. (1994) *Trends Pharmacol. Sci.* **15,** 114–119
9. Takenaka, T., Forster, H., and Epstein, M. (1993) *Circ. Res.* **73,** 743–750
10. Scholz, H., and Kurtz, A. (1990) *Am. J. Physiol.* **259,** C421–C426
11. Purdy, R. E., and Weber, M. A. (1988) *Circ. Res.* **63,** 748–757
12. Dosemeci, A., Dhallan, R. S., Cohen, N. M., Lederer, W. J., and Rogers, T. B. (1988) *Circ. Res.* **62,** 347–357
13. Macrez-Lepretre, N., Morel, J. L., and Mironneau, J. (1996) *J. Pharmacol. Exp. Ther.* **278,** 468–475
14. Benham, C. D., Bolton, T. B., and Lang, R. J. (1985) *Nature* **316,** 345–347
15. Shi, X. Z., and Sarna, S. K. (2000) *Am. J. Physiol.* **278,** G234–G242
16. Liu, X., Rusch, N. J., Striessnig, J., and Sarna, S. K. (2001) *Gastroenterology* **120,** 480–489
17. Bauer, J., Dau, C., Cavarape, A., Schaefer, F., Ehmke, H., and Parekh, N. (1999) *Am. J. Physiol.* **277,** H1–H7
18. Nagahama, T., Hayashi, K., Ozawa, Y., Takenaka, T., and Saruta, T. (2000) *Kidney Int.* **57,** 215–223
19. Lambert, C. (1995) *Br. J. Pharmacol.* **115,** 795–800
20. Fan, Q. I., Vanderpool, K., and Marsh, J. D. (2002) *Biochim. Biophys. Acta* **1577,** 401–411
21. Kato, H., Hayashi, T., Koshino, Y., Kutsumi, Y., Nakai, T., and Miyabo, S. (1992) *Biochem. Biophys. Res. Commun.* **188,** 934–941
22. Savignac, M., Badou, A., Moreau, M., Leclerc, C., Guery, J. C., Paulet, P., Druet, P., Ragab-Thomas, J., and Pelletier, L. (2001) *FASEB J.* **15,** 1577–1579
23. Fish, R. D., Sperti, G., Colucci, W. S., and Clapham, D. E. (1988) *Circ. Res.* **62,** 1049–1054
24. Lacerda, A. E., Rampe, D., and Brown, A. M. (1988) *Nature* **335,** 249–251
25. Tseng, G. N., and Boyden, P. A. (1991) *Am. J. Physiol.* **261,** H364–H379
26. Liu, Q. Y., Karpinski, E., and Pang, P. K. (1993) *Biochem. Biophys. Res. Commun.* **191,** 796–801
27. Schuhmann, K., and Groschner, K. (1994) *FEBS Lett.* **341,** 208–212
28. Chik, C. L., Li, B., Ogiwara, T., Ho, A. K., and Karpinski, E. (1996) *FASEB J.* **10,** 1310–1317
29. Reeve, H. L., Vaughan, P. F., and Peers, C. (1995) *Pfluegers Arch. Eur. J. Physiol.* **429,** 729–737
30. Love, J. A., Richards, N. W., Owyang, C., and Dawson, D. C. (1998) *Am. J. Physiol.* **274,** G397–G405
31. Boixel, C., Tessier, S., Pansard, Y., Lang-Lazdunski, L., Mercadier, J. J., and Hatem, S. N. (2000) *Am. J. Physiol.* **278,** H670–H676
32. Zhang, Z. H., Johnson, J. A., Chen, L., El-Sherif, N., Mochly-Rosen, D., and

Boutjdir, M. (1997) *Circ. Res.* **80,** 720–729
33. Watts, S. W., Florian, J. A., and Monroe, K. M. (1998) *J. Pharmacol. Exp. Ther.* **286,** 1431–1438
34. Seki, T., Yokoshiki, H., Sunagawa, M., Nakamura, M., and Sperelakis, N. (1999) *Pfluegers Arch. Eur. J. Physiol.* **437,** 317–323
35. Quignard, J. F., Mironneau, J., Carricaburu, V., Fournier, B., Babich, A., Nurnberg, B., Mironneau, C., and Macrez, N. (2001) *J. Biol. Chem.* **276,** 32545–32551
36. Bouron, A., Soldatov, N. M., and Reuter, H. (1995) *FEBS Lett.* **377,** 159–162
37. Oz, M., Melia, M. T., Soldatov, N. M., Abernethy, D. R., and Morad, M. (1998) *Mol. Pharmacol.* **54,** 1106–1112
38. Snutch, T. P., Tomlinson, W. J., Leonard, J. P., and Gilbert, M. M. (1991) *Neuron* **7,** 45–57
39. Mikami, A., Imoto, K., Tanabe, T., Niidome, T., Mori, Y., Takeshima, H., Narumiya, S., and Numa, S. (1989) *Nature* **340,** 230–233
40. Biel, M., Ruth, P., Bosse, E., Hullin, R., Stuhmer, W., Flockerzi, V., and Hofmann, F. (1990) *FEBS Lett.* **269,** 409–412
41. Koch, W. J., Ellinor, P. T., and Schwartz, A. (1990) *J. Biol. Chem.* **265,** 17786–17791
42. Shistik, E., Ivanina, T., Blumenstein, Y., and Dascal, N. (1998) *J. Biol. Chem.* **273,** 17901–17909
43. Shistik, E., Keren-Raifman, T., Idelson, G. H., Blumenstein, Y., Dascal, N., and Ivanina, T. (1999) *J. Biol. Chem.* **274,** 31145–31149
44. Blumenstein, Y., Kanevsky, N., Sahar, G., Barzilai, R., Ivanina, T., and Dascal, N. (2002) *J. Biol. Chem.* **277,** 3419–3423
45. Dai, B., Saada, N., Echetebu, C., Dettbarn, C., and Palade, P. (2002) *Biochem. Biophys. Res. Commun.* **296,** 429–433
46. Soldatov, N. M. (1994) *Genomics* **22,** 77–87
47. Dascal, N., and Lotan, I. (1992) in *Protocols in Molecular Neurobiology*, Vol. 13, pp. 205–225, Humana Press, Totowa, NJ
48. Shistik, E., Ivanina, T., Puri, T., Hosey, M., and Dascal, N. (1995) *J. Physiol. (Lond).* **489,** 55–62
49. Kanki, H., Kinoshita, M., Akaike, A., Satoh, M., Mori, Y., and Kaneko, S. (2001) *Mol. Pharmacol.* **60,** 989–998
50. Sharon, D., Vorobiov, D., and Dascal, N. (1997) *J. Gen. Physiol.* **109,** 477–490
51. Singer, D., Biel, M., Lotan, I., Flockerzi, V., Hofmann, F., and Dascal, N. (1991) *Science* **253,** 1553–1557
52. Soldatov, N. M., Bouron, A., and Reuter, H. (1995) *J. Biol. Chem.* **270,** 10540–10543
53. Doan, T. N., Ali, M. S., and Bernstein, K. E. (2001) *J. Biol. Chem.* **276,** 20954–20958
54. Dascal, N. (1987) *CRC Crit. Rev. Biochem.* **22,** 317–387
55. Pitt, G. S., Zuhlke, R. D., Hudmon, A., Schulman, H., Reuter, H., and Tsien, R. W. (2001) *J. Biol. Chem.* **276,** 30794–30802
56. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., and Loriolle, F. (1991) *J. Biol. Chem.* **266,** 15771–15781
57. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993) *J. Biol. Chem.* **268,** 9194–9197
58. Hill, M. M., Andjelkovic, M., Brazil, D. P., Ferrari, S., Fabbro, D., and Hemmings, B. A. (2001) *J. Biol. Chem.* **276,** 25643–25646
59. Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., and Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135,** 397–402
60. Zamponi, G. W., Bourinet, E., Nelson, D., Nargeot, J., and Snutch, T. P. (1997) *Nature* **385,** 442–446
61. McHugh, D., Sharp, E. M., Scheuer, T., and Catterall, W. A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 12334–12338
62. Newton, A. C. (1997) *Curr. Opin. Cell Biol.* **9,** 161–167
63. Singer-Lahat, D., Gershon, E., Lotan, I., Hullin, R., Biel, M., Flockerzi, V., Hofmann, F., and Dascal, N. (1992) *FEBS Lett.* **306,** 113–118