JPET Fast Forward. Published on July 31, 2006 as DOI: 10.1124/jpet.106.107607

JPET #107607

Voltage-gated K^+ channel block by catechol derivatives: defining nonselective and selective pharmacophores

Vicenta Salvador-Recatala, Yonjung Kim, Elena Zaks-Makhina, and Edwin S. Levitan

Department of Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261, USA

Running title: Kv channel block by catechol and derivatives

Address correspondence to: Edwin S. Levitan, PhD, Department of Pharmacology, University of Pittsburgh, Pittsburgh, PA 15261, USA. Phone: 412-648-9486. Fax: 412-648-1945. email: Levitan@server.pharm.pitt.edu

18 text pages

6 figures

0 tables

20 references

Abstract: 229 words

Introduction: 464 words

Discussion: 607 words

NONSTANDARD ABBREVIATIONS:

TEA, tetraethylammonium

48F10, 3-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol

4-Nb-cat, 4-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol

Recommended section: Cellular and Molecular

Abstract

High throughput screening led to the identification of a 3-norbornyl derivative of catechol called 48F10 as a Kv2.1 K^+ channel inhibitor. By virtue of the involvement of Kv2.1 channels in programmed cell death, 48F10 prevents apoptosis in cortical neurons and enterocytes. This uncharged compound acts with an apparent affinity of 1 μM at the tetraethylammonium (TEA) site at the external mouth of the Kv2.1 channel, but is ineffective on Kv1.5. Here we investigate the basis of this selectivity with structure-activity studies. We find that catechol (1,2-benzenediol), unlike 48F10, inhibits Kv2.1 currents with a Hill coefficient of 2 and slows channel activation. Furthermore, this inhibition, which requires millimolar concentrations, is unaffected by external TEA or by mutation of the external tyrosine implicated in channel block by TEA and 48F10. Also, catechol does not distinguish between Kv2.1 and Kv1.5. Thus, catechol acts at conserved sites that are distinct from 48F10. We also tested 11 catechol derivatives based on hydrocarbon adducts including norbornyl substructures, a 48F10 isomer and a 48F10 diastereomer. These compounds are more potent than catechol, but none recapitulated 48F10's marked selectivity Kv2.1 over Kv1.5. We conclude that targeting of 48F10 to the TEA site at the external mouth of the Kv2.1 pore and away from other sites involved in nonselective Kv channel block by catechol requires the norbornyl group in a unique position and orientation on the catechol ring.

Introduction

Voltage-gated K^+ (Kv) channels comprise a large superfamily of genes that influence the function of excitable cells. Furthermore, in many cell types these channels are required for programmed cell death (Yu, 2003). For example, an increase in Kv2.1 channel activity, mediated by trafficking to the cell surface, has been implicated in apoptosis of cortical neurons and enterocytes (Pal et al. 2003, 2006; Zaks-Makhina et al., 2004; Grishin et al., 2005). The diversity and importance of Kv channels suggests that they could be useful therapeutic targets. Indeed, aminopyridines have been used to promote nerve conduction in multiple sclerosis and it is thought that cardiac Kv channel blockers could be useful as class 3 antiarrhythmic drugs. However, a major challenge to development of Kv channel drugs is conservation of the pore, which makes development of selective compounds difficult.

Recently, a high throughput yeast-based screening strategy led to the identification of a new K^+ channel blocker called 48F10 (3-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol, see Fig. 1A for structure) (Zaks-Makhina et al., 2004). This norbornyl-catechol compound is selective for Kv2.1 over Kir2.1 and Kv1.5 channels. A combination of site directed mutagenesis and pharmacological experiments showed that 48F10, although uncharged, acts at the external mouth of the pore at a site that overlaps with the tetraethlyammonium (TEA) block site. Strikingly, the affinity of 48F10 was ~5000-fold greater than TEA for Kv2.1. In addition, micromolar doses of 48F10 are neuroprotective because they block Kv2.1-mediated apoptosis of cortical neurons (Zaks-Makhina et al., 2004). More recently, 48F10 has been shown to prevent Kv2.1-mediated apoptosis in intestinal enterocytes (Grishin et al., 2005). Thus, experiments

with this compound suggest that Kv channel blockers could be developed for therapeutic protection from apoptosis in a wide variety of tissues.

The basis for the high affinity and selectivity of 48F10 for Kv2.1 channels is unknown. Catechol (i.e., 1,2-benzenediol), which is a moiety of 48F10 (see Fig. 1A and B for structures), blocks native Kv channels (Ito and Maeno, 1986; Erdelyi and Such, 1988; Kuenzi and Dale, 1998; Hess and El Manira, 2001), but its action has not been studied on Kv2.1 or any other cloned Kv channels. It is known, however, that appending hydrophobic hydrocarbon groups to TEA greatly increases affinity for the internal pore (Armstrong, 1971). Therefore, we hypothesized that the norbornyl moiety in 48F10 simply enhances the affinity of catechol for Kv2.1. To explore this hypothesis, we examined the block of Kv2.1 and Kv1.5 channels by catechol and a series of catechol derivatives. These studies first show that catechol acts at different sites from 48F10. Furthermore, none of the tested compounds, including an isomer and a diastereomer of 48F10, are markedly selective for Kv2.1 over Kv1.5. Therefore, 48F10 represents a unique pharmacophore that is distinguished from less specific catechol derivatives by the location and orientation of the norbornyl moiety.

Methods

Whole cell patch clamp recordings were performed with 1 to 3 megohm electrodes on CHO cells expressing Kv2.1 or Kv1.5 similarly to a previous study (Zaks-Makhina et al., 2004). Typically, series resistance was <10 megohm and compensated by $\geq$70%. For Kv1.5, channel expression plasmids were cotransfected with an EGFP expression vector by lipofection with lipofectamine 2000 (Invitrogen) or Tfx-50

(Promega). For Kv2.1, the same approach was used for transient transfection or a stably transfected cell line was used (Trapani and Korn, 2003). Currents were evoked by voltage steps from -60 to +40 mV unless otherwise indicated. Drugs were delivered using a gravity fed micro-superfusion system. In all cases, the onset and reversal of drug effects appeared to reflect limitations in the kinetics of solution changes. Commercially available compounds (Fig. 1) were purchased from Sigma-Aldrich or Chembridge. Fluorous Technologies synthesized the nine compounds in Figure 6 with an ortholithiation-deoxygenation procedure based on previous publications (Green et al., 2000; Adlington et al., 1976; McOmie et al., 1973): (Scheme 1)

Structures were verified by NMR.

Results

A soluble carboxylic acid adduct of norbornane (exo-bicyclo(2.2.1)heptane-2-carboxylic acid, see Fig. 1C for structure) does not inhibit Kv2.1 channels at a concentration of 100 μM (data not shown), while catechol is a known K^+ channel inhibitor. To test the hypothesis that the norbornyl moiety in 48F10 simply increases the potency of the low affinity blocker catechol, we characterized catechol action on heterologously expressed Kv2.1 channels. Voltage clamp measurements show that catechol acts at millimolar doses to inhibit Kv2.1 channel activity (Fig. 2A). This inhibition displays many features that are not evident with 48F10. First, catechol alters Kv2.1 gating: the kinetics and voltage dependence of channel activation are shifted (Fig. 2B,C). Second, the dose-response relationship reveals cooperative inhibition: the

Hill coefficient for catechol is 2.1 (Fig. 2D). Third, catechol inhibition of Kv2.1 channels is unaffected by the presence of 20 mM external TEA (tetraethylammonium) (Fig. 2E,F). At this concentration, TEA inhibits 82 $\pm$ 6 % of the Kv2.1 current (data not shown). The lack of a shift in sensitivity to catechol in the presence of significant TEA-mediated block suggests that catechol does not act at an overlapping site at the external mouth of the channel with TEA. Thus, catechol is mechanistically distinct from 48F10.

Inhibition of Kv1.5, a channel that is insensitive to 48F10 and TEA, further distinguished catechol from 48F10. First, while 48F10 is much less effective with Kv1.5 than Kv2.1 channels, catechol inhibition occurs with an EC50 value of 3 mM for both Kv2.1 (Fig. 2A,D) and Kv1.5 (Fig. 3A,B). Furthermore, catechol inhibition of Kv1.5 is unaffected by the R476Y mutation (Fig. 3B,C), which confers sensitivity to this channel to external TEA and 48F10 . Therefore, catechol appears to inhibit Kv1.5 channels at a site distinct from the extracellular TEA binding site on the R476Y mutant (Fedida et al., 1999; Zaks-Makhina et al., 2004). The results in Figures 2 and 3 indicate that the norbornyl moiety on the α carbon (i.e. carbon 3) of catechol does not simply increase affinity. Instead, it is part of a pharmacophore that interacts with a specific site on external pore of Kv2.1. In contrast, catechol acts at distinct sites present on Kv1.5 and Kv2.1.

These findings raise the question of whether any hydrophobic catechol adduct would act similarly to 48F10. Therefore, we examined 4-tert-octyl-catechol (see structure in Fig. 1D). As can be seen in Figure 4A-C, this derivative is a micromolar affinity inhibitor of Kv2.1 that acts with a Hill coefficient of 1. These features are comparable to 48F10. However, 4-tert-octyl-catechol inhibition of Kv2.1 was not

reduced significantly by external TEA (Fig. 4C,D,E), showing that this catechol derivative does not act at the external TEA binding site. Furthermore, 4-tert-octyl-catechol avidly inhibited Kv1.5, and this inhibition was unaffected by the R476Y mutation (Figure 4F,G,H): EC50 values equaled ~4 μM for the wild type channel and the external pore mutant. Thus, appending a hydrophobic aliphatic group to catechol increased its affinity and reduced cooperativity, but did not induce the selectivity for the Kv2.1 external pore 48F10/TEA site.

This result could reflect one of two differences between 48F10 and 4-tert-octyl-catechol: the location on the catechol ring of the hydrophobic derivative (i.e., catechol carbon 4 for the tert-octyl derivative versus catechol carbon 3 position for 48F10) or the moieties themselves (tert-octyl versus norbornyl). To discriminate between these possibilities, we examined the action of a 48F10 isomer (4-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol), which contains the same norbornyl moiety as 48F10, but located on carbon 4 instead of carbon 3 (see Fig. 1E for structure). 20 μM of this compound (here called 4-Nb-cat) partially inhibited Kv2.1 channels (Fig. 5A, left), indicating lower potency than 48F10. More importantly, Kv2.1 inhibition was unaffected by 20 mM external TEA (Fig. 5A, right) and this isomer was similarly effective on Kv1.5 (Fig. 5B) and R476Y Kv1.5 mutant channels (Fig. 5C). Also, block of Kv2.1 was cooperative (data not shown). Thus, the carbon 4 norbornyl isomer does not have the selectivity associated with the 48F10 external binding site. Rather, it behaves more like catechol.

We next examined the activity of nine 3-position adducts. Specifically, we tested moieties that have similar hydrophobicity to 48F10 or represented substructures of the norbonyl adduct in 48F10 (Fig. 6). All of these compounds inhibited Kv2.1 (filled

circles), with affinities in the range of 10 to 200 μM. Furthermore, Kv2.1 inhibition occurred with a Hill coefficient of 2 and slowed Kv2.1 activation in all cases (data not shown), as is the case with catechol. Finally, we found that each of these compounds inhibited Kv1.5 (open circles). The latter action displayed potencies similar to or higher than for block of Kv2.1 channels. Indeed, the only compound to show any preference for Kv2.1 channels over Kv1.5 channels was a diastereomer of 48F10 (Fig. 6, bottom panel), but the difference in affinities for the two channels was modest (i.e., ~3-fold difference in EC50). These results imply that the marked selectivity of 48F10 for the external pore of Kv2.1 requires a complete norbornyl moiety appended to the 3-position of catechol in a specific orientation.

Discussion

High throughput screening led to the discovery of the norbornyl-catechol derivative 48F10, which selectively inhibits Kv2.1 channels with an EC50 of 1 μM (Zaks-Makhina et al., 2004). By virtue of this inhibition, this compound prevents apoptosis mediated by Kv2.1 channels in neurons and enterocytes (Zaks-Makhina et al., 2004; Grishin et al., 2005). Structure function and pharmacological experiments revealed that 48F10 acts at the TEA site of the external mouth of the channel pore (Zaks-Makhina et al., 2004). Here we set out to test the hypothesis that this compound simply represents a high affinity version of catechol, a known blocker of native Kv channels. However, our results demonstrate that catechol action is fundamentally different than 48F10: it is not selective for Kv2.1 over Kv1.5, does not bind to the external TEA site, acts cooperatively, and alters activation. Tests with a series of catechol derivatives indicate

that appending hydrocarbon groups to catechol increases affinity roughly in proportion to hydrophobicity and reduces cooperativity, but does not mimic the selectivity of 48F10 for Kv2.1 over Kv1.5. Indeed, we showed that the norbornyl moiety must be appended to the catechol ring in the right position (i.e., carbon 3) and orientation to selectively target the Kv2.1 external TEA site.

These results raise the question of how an uncharged molecule like 48F10 can bind at the external mouth of the Kv2.1 pore and compete with TEA. The TEA binding to the 48F10-sensitive channels studied here (i.e. wild type Kv2.1 and R476Y Kv1.5) has been thought to be mediated directly by a tyrosine. Indeed, a similar interaction with a bacterial channel has been modeled with molecular dynamics (Crouzy et al., 2001; Guidoni and Carloni, 2002; Luzhkov et al., 2003) and studied by crystallization with TEA analogs (Lenaeus et al., 2005). However, site directed mutagenesis and chemical modification results have called this model into question for Kv2.1: although the tyrosine side chain clearly has a role in external TEA block, it may not cage or bind TEA stably or directly in mammalian Kv2.1 channels (Andalib et al., 2004). The uncertainty in the mechanism of TEA block of Kv2.1 makes interpretation of the competition between TEA and 48F10, but not catechol and many derivatives, difficult to interpret in structural terms. One possible hypothesis is that the hydroxyl groups on the catechol ring interact with carbonyls on the peptide backbone of the channel. With 48F10, the catechol group may interact with the side of the channel mouth and position the norbornyl group, which is comparable in size to the selectivity filter, in the conduction pathway. Alternatively, the hydrophobic norbornyl group could interact with hydrophobic moieties in the external mouth to position catechol in the conduction

pathway. It is also possible that 48F10 interacts with TEA-interacting tyrosines more directly via hydrophobic and or pi-pi interactions (i.e., the phenyl rings in of tyrosine and catechol could stack) to block the extracellular vestibule. With any of the above models, block would not be expected to be voltage dependent, or subject of "knock off" by conducting K^+ because 48F10 is uncharged.

For catechol itself, it is conceivable that two molecules must bind to the tetrameric channel to cooperatively occlude the conduction pathway. However, the marked effects on the kinetics and voltage dependence of Kv2.1 activation by catechol and many of its derivatives suggest an interaction with the gating mechanism. This would not be expected for a simple pore blocker. In fact, internal TEA does not appear to compete with 4-Nb-cat inhibition of Kv2.1 (unpublished results), excluding one known conventional blocking site. Nevertheless, the nonspecific interactions of catechols with Kv channels could be based on binding to another pore site that acts allosterically to influence gating.

Acknowledgments

We are grateful to Dr. John Lazo and members of the University of Pittsburgh Drug Discovery Institute for their advice and assistance with compound screening that led to this study.

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Footnotes

Vicenta Salvador-Recatala and Yonjung Kim contributed equally. This research was supported by NIH grants R01NS32385, R01HL55312 and R01HL80632 to ESL and R21NS048089 to EZ-M.

Legends for Figures

Figure 1. Structures of 48F10, catechol and other commercially available compounds used in this study. A. 48F10. B. Catechol. C. exo-bicyclo(2.2.1)heptane-2-carboxylic acid D. 4-tert-octyl-catechol. E. 4-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol (4-Nb-cat).

Figure 2. Inhibition of Kv2.1 channels by catechol. A. Effect of catechol on Kv2.1 currents evoked by a voltage step to 40 mV. B. Catechol slows activation. Inset, normalized currents evoked by a voltage step to 50 mV from -70 mV in the absence (filled circle) and presence of 3 mM catechol (open circle). Main graph, catechol shifts the relationship between membrane potential (Vm) and the time constant of activation. Control (filled circles), N=6; 3 mM catechol (open circles), N=6; 10 mM catechol (open squares), N=5. C. Normalized conductance (G/Gmax) versus membrane potential (Vm) in the absence (filled circles, N=6) and presence of 3 (open circles, N=6) and 10 mM (open squares, N=5) catechol. N=5. D. Dose-response relationship for catechol. N=6-10 for each point. The fitted curve has a Ki of 3 mM and a Hill coefficient of 2.1. E. Current traces showing that catechol remains effective in the continued presence of 20 mM extracellular TEA. F. Quantitation of block by 3 mM catechol in the presence and absence of TEA. N=13.

Figure 3. Inhibition of Kv1.5 channels by catechol. A. Effect of catechol of Kv1.5 currents evoked by a voltage step to 40 mV. B. Quantitation of the catechol effect on wild type and R476Y mutant Kv1.5 channels. N = 5 for each point. Curve shows Ki of 3

mM and Hill slope of 1.35. C. Effect of catechol on R476Y mutant Kv1.5 channels.

Figure 4. Block of Kv2.1 and Kv1.5 channels by 4-tert-octyl-catechol. A. Block of Kv2.1 currents evoked by a voltage step to 40 mV. B. Dose-response curve for Kv2.1 channels. N=6-11 for each point. Fitted curve has a Ki of 2.1 mM and a Hill coefficient of 1. C, D. Block by 2 μM drug is unaffected by 20 mM TEA. E. Quantitation of the interaction between TEA and 4-tert-octyl-catechol. N=9. F. Block of wild type Kv1.5 currents evoked by a voltage step to 40 mV. G. Block of R476Y mutant Kv1.5 currents evoked by a voltage step. G. Quantitation of the drug effect on wild type and mutant Kv1.5 channels. N=5 for each point. The curve is drawn with a Ki=4 μM and a Hill coefficient of 1.

Figure 5. Effects of the 4-norbornyl-catechol (4-Nb-cat) derivative 4-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol on Kv2.1 and Kv1.5. A. Percentage inhibition of Kv2.1 current by 20 μM 4-Nb-cat in the presence or absence of 20 mM TEA. B. Inhibition of wild type Kv1.5 by 25 μM 4-Nb-cat. C. Inhibition of Kv1.5 R486Y by 25 μM 4-Nb-cat. Note that 4-Nb-cat inhibits wild type Kv1.5 at least as well as mutant channels, while the 3-norbornyl-catechol compound 48F10 is >100-fold more effective on the mutant.

Figure 6. Effects of nine 3-position catechol derivatives on Kv2.1 (closed circles) and Kv1.5 (open circles) channels. The structure of each compound is shown with dose-response curves. Peak currents evoked by voltage pulses from -70 to +70 mV were measured.

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Scheme 1

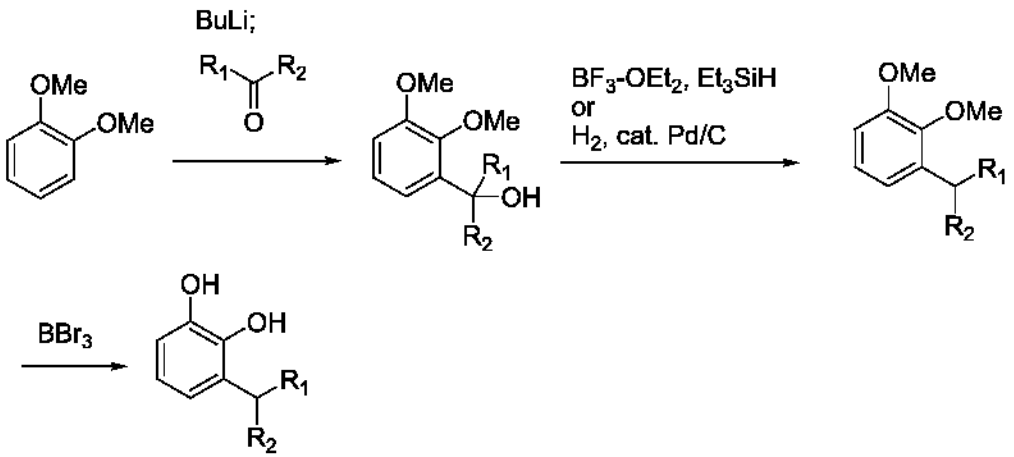

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Figure 1

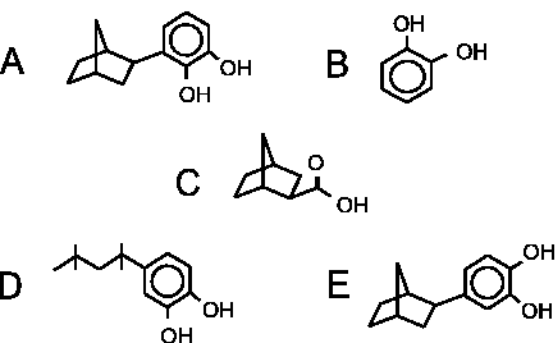

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Figure 2

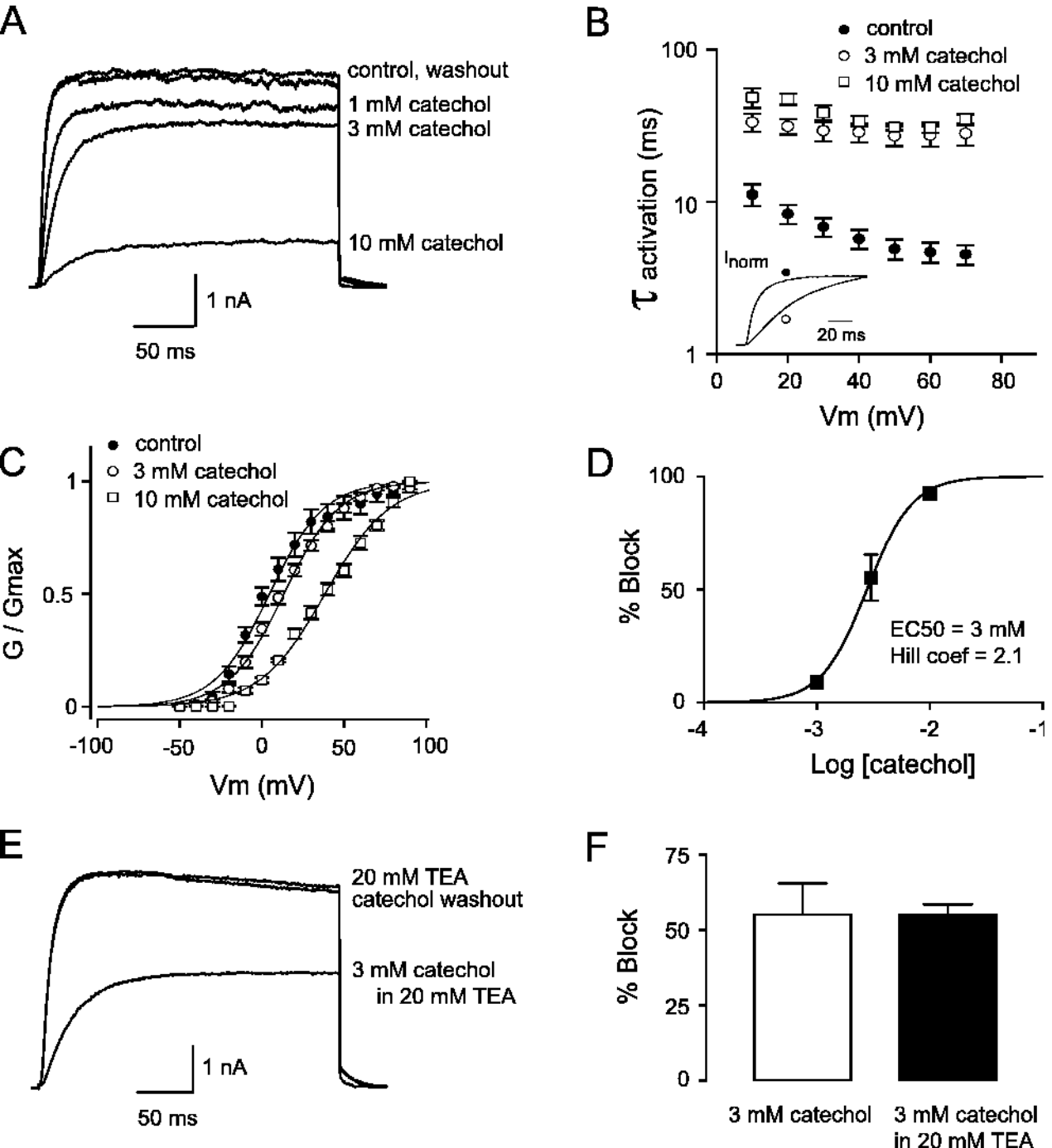

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Figure 3

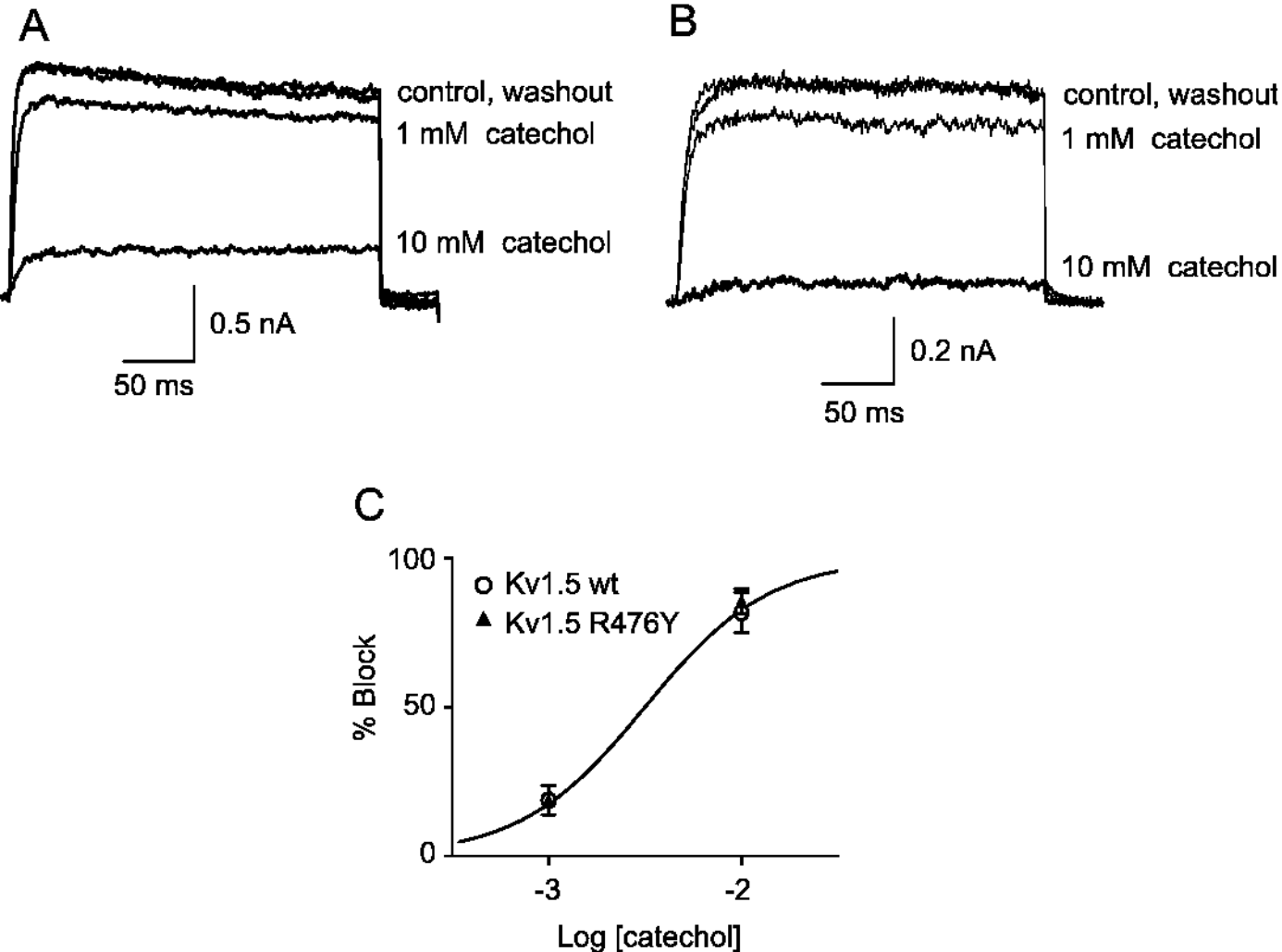

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Figure 4

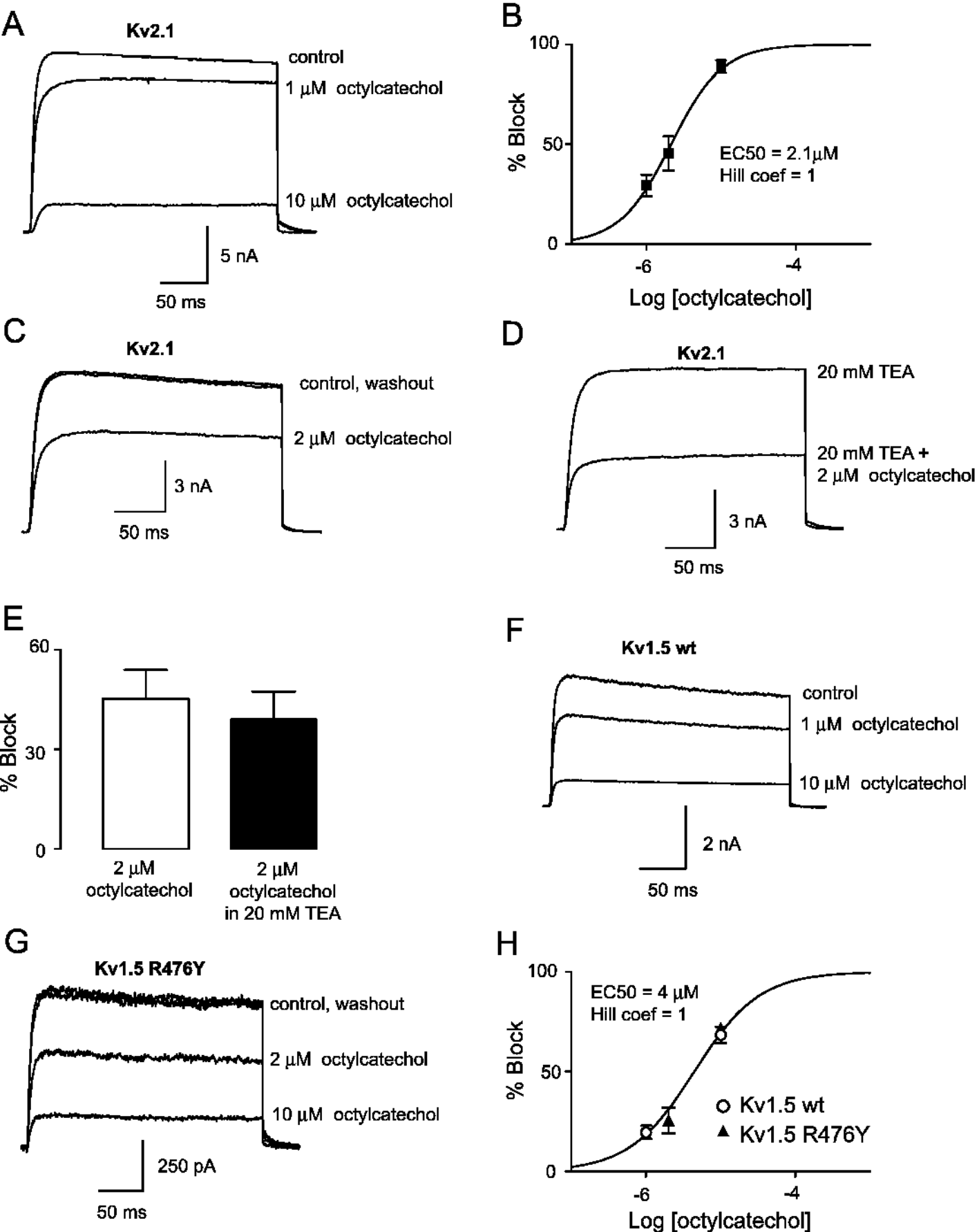

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Figure 5

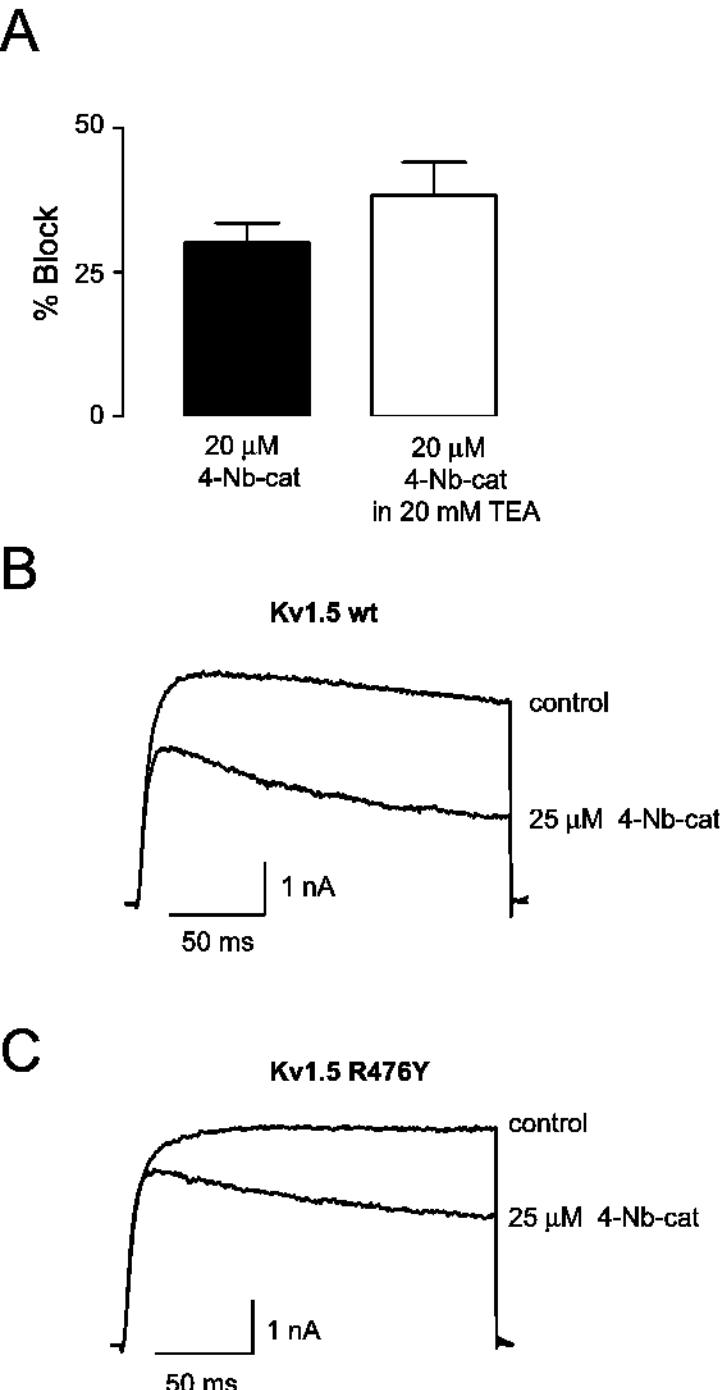

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Figure 6