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Effects of Antidepressants on G Protein–coupled Receptor Signaling and Viability in Xenopus laevis *Oocytes*

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Background: **Tricyclic antidepressants are structurally related to local anesthetics, suggesting that part of their analgesic action may result from properties shared with local anesthetics. Because local anesthetics block G protein–coupled receptor signaling (which explains, in part, their inflammatory modulating properties), the authors studied whether antidepressants have similar effects.**

Methods: **Peak Ca-activated Cl currents induced in *Xenopus laevis* oocytes by lysophosphatidic acid (10^{-4} M) were measured using a voltage clamp. The effects of a 30-, 120-, or 240-min incubation in amitriptyline, nortriptyline, imipramine, or fluoxetine were determined.**

Results: **After a 30-min incubation, low concentrations (10^{-7}–10^{-5} M) of antidepressants had no effect on lysophosphatidic acid–induced currents. After prolonged incubation, only amitriptyline or nortriptyline inhibited lysophosphatidic acid signaling (each to 58% of the control response at 10^{-7} M after 240 min). At low concentrations, none of the compounds induced membrane damage (defined as a holding current of > 1 μA, 2% in control cells). Imipramine at 10^{-3} M induced damage in 100% of oocytes, and fluoxetine at 10^{-4} M induced damage in 71% of oocytes ($P < 0.05$ *vs*. control). Amitriptyline and nortriptyline had no effect.**

Conclusions: **These findings are in part different from those obtained with local anesthetics and suggest that interference with G protein–coupled signaling might explain, in part, the analgesic properties of some antidepressants. However, use of antidepressants in high concentrations may be associated with cellular toxicity.**

THE tricyclic antidepressant (TCA) amitriptyline is used frequently in the treatment of chronic pain,[1–3] but the mechanism of its action is not understood. This limits further development of TCA for use in chronic pain therapy. One potential clue to the mechanism of action of amitriptyline might be found in the observation that TCAs share several structural similarities with local anesthetics (LAs). Both groups of compounds consist of a hydrophobic portion (usually a single-ring structure in LAs and a tricyclic structure in TCAs) linked *via* a linear intermediate moiety (an amide or ester linkage in LAs and a hydrocarbon chain in TCAs) to an amide. Amitriptyline also shares some of the functional properties of LAs: the compound is known to have sodium channel blocking properties[4] and is therefore under investigation for use as a long-acting LA.[5,6] Conversely, LA infusion is of benefit for the treatment of some forms of chronic pain.[7] These similarities in structure and effects suggest that the beneficial effects of TCAs in the treatment of chronic pain might result, in part, from some properties they share with LAs.

One property of LAs that recently has received attention is their ability to inhibit G protein–coupled signaling and thereby modulate the inflammatory response.[8] Because many chronic pain syndromes have an inflammatory component,[9–11] an inflammatory modulating effect could potentially explain part of the clinical efficacy of LAs in these settings. Because of the structural similarities with LAs, we hypothesized that TCAs might act in a similar manner and inhibit G protein–coupled signaling.

To test this hypothesis, we determined, using *Xenopus* oocytes, the effects of TCAs on receptors for lysophosphatidic acid (LPA). LPA receptors were chosen because they have been studied extensively as targets for LA sensitivity. Oocyte LPA signaling was shown to be inhibited by a number of clinically relevant LAs,[12] and several interacting binding sites for charged and uncharged LAs were identified.[13] In human neutrophils, LPA-induced priming is inhibited by LAs.[14] LPA signaling inhibition by LAs results in part from selective inhibition of Gq protein function, which has been demonstrated for both *Xenopus*[15] and mouse[16] Gq protein. Importantly, LA inhibition of LPA signaling is profoundly time-dependent: 5- to 10-fold greater LA potency is attained after incubation for hours to days, and after such time frames, inhibition is observed at micromolar concentrations. Recently, we showed that this time-dependent effect is also mediated, in part, by an action on Gq signaling.[17] Thus, the pharmacology of LA interactions with LPA signaling is well established. In addition, these receptors may be potentially relevant to pain medicine, because LPA is a putative wound healing and inflammatory mediator[18] and is involved in peripheral pain transmission.[19,20]

We therefore determined the effects on LPA signaling of amitriptyline, the TCA used primarily for pain therapy in the clinical setting, and compared its effects with those of two other TCAs (nortriptyline and imipramine)

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as well as one antidepressant with a different structure (fluoxetine).

Materials and Methods

The University of Maastricht Animal Care and Use Committee approved the study protocols.

Xenopus laevis *Oocyte Electrophysiology*

Oocyte harvesting and electrophysiologic recording were performed as described previously.[21] Briefly, oocytes were obtained from *X. laevis* toads and defolliculated with collagenase. Ca-activated Cl currents (induced by intracellular Ca-release due to LPA receptor–mediated inositol 1,4,5-triphosphate generation and subsequent intracellular Ca release) were measured using a two-electrode voltage clamp. Holding currents of greater than 1 μA were considered to indicate membrane damage. In cells with membrane damage, Cl currents could not be determined; such cells were therefore not included in the analysis. Only a single measurement was obtained in each cell.

Study Protocol

The effects of a 30-, 120-, or 240-min preincubation with nortriptyline (10^{-7}–10^{-4} M), amitriptyline (10^{-7}–10^{-3} M), imipramine (10^{-7}–10^{-3} M), or fluoxetine (10^{-7}–10^{-4} M) (all from Sigma Chemical Company, St. Louis, MO) on peak Cl currents induced by LPA (10^{-4} M; Avanti Polar Lipids, Alabaster, AL) were determined. TCAs were dissolved in Tyrode buffer (containing NaCl, 150 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; dextrose, 10 mM; and HEPES, 10 mM; pH adjusted to 7.4) as the vehicle. Tyrode solution alone did not have an effect on LPA responses or membrane integrity (data not shown). For determination of the site of action, oocytes were injected with the protein kinase C (PKC) inhibitor chelerythrine (Sigma Chemical Company) in an estimated final concentration of 5×10^{-5} M 2 h before experimentation. To determine the effect of the TCA on the endogenous oocyte Ca-activated Cl channel, we activated the Cl channel directly by injecting $CaCl_2$ (30 nl) into the oocyte (estimated final concentration, 10^{-5} M) under voltage clamp.

Statistical Analysis

Data are mean ± SD. In the figures, membrane damage induced by TCAs is reported as the difference between the percentage of cells damaged in the TCA group and the percentage of cells damaged in the corresponding control group (*i.e.*, excess damage induced by TCAs). LPA-induced peak Cl currents in *X. laevis* oocytes are reported in microamperes. Measurements in at least 20 oocytes were averaged to generate each data point. Because of the variability between oocyte batches, responses were at times normalized to the control. Statistical analysis was performed using the Student *t* test or one-way ANOVA followed by the Tukey test, as appropriate. $P < 0.05$ was considered significant.

Results

LPA Signaling in X. laevis *Oocytes*

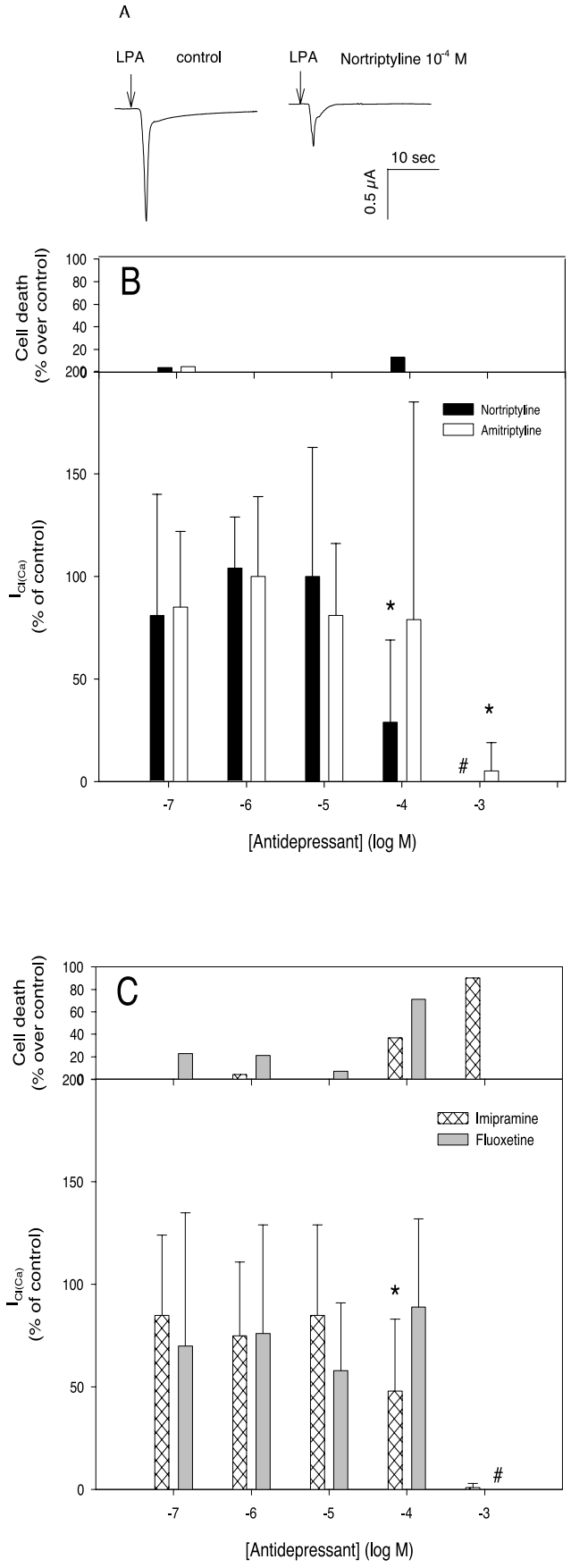
LPA at 10^{-4} M induced inward currents in oocytes, with an average peak current of 2.1 μA (fig. 1A). The currents had the typical shape of Ca-activated Cl currents in this model, and we have shown previously that these currents are dependent on intracellular Ca release induced by inositol 1,4,5-triphosphate.[12]

Short-term Effects of TCAs on LPA Signaling in X. laevis *Oocytes*

We then studied the effects of a 30-min pretreatment with nortriptyline, amitriptyline, imipramine, or fluoxetine on currents induced by LPA at 10^{-4} M. The effects observed depended greatly on the TCA used, and results divided naturally into two groups: those obtained with amitriptyline or nortriptyline and those obtained with imipramine or fluoxetine.

Amitriptyline and Nortriptyline. At concentrations between 10^{-7} and 10^{-5} M, oocytes treated with amitriptyline or nortriptyline showed responses not significantly different from those observed in control cells (fig. 1B). At greater concentrations, these compounds induced membrane damage in a small percentage (< 15%) of cells (fig. 1B). In the nondamaged cells, we assessed the effects of the compounds on peak currents induced by LPA. Whereas 10^{-5} M nortriptyline did not depress LPA signaling, 10^{-4} M inhibited responses to 29% of the control (fig. 1A and B). Amitriptyline at 10^{-4} M did not have an effect on peak currents, but 10^{-3} M inhibited LPA-induced Cl currents to 5% of the control (fig. 1B).

Imipramine and Fluoxetine. At concentrations between 10^{-7} and 10^{-5} M, oocytes treated with imipramine or fluoxetine showed responses not significantly different from those observed in control cells (fig. 1C). At greater concentrations, the effects of the compounds were quite different from those observed using amitriptyline or nortriptyline. Remarkably, imipramine (10^{-4} and 10^{-3} M) or fluoxetine (10^{-4} M) were toxic to oocytes: 100% of the imipramine-incubated cells and 71% of the fluoxetine-incubated cells showed membrane damage (compared with < 10% in control cells; fig. 1C). Because all cells exposed to imipramine were damaged, we could not assess its effect on LPA signaling. We tested the effect of fluoxetine on LPA-induced currents in those cells not damaged by the TCA and found no effect.



Long-term Effects of TCAs on LPA Signaling in X. laevis *Oocytes*

LA inhibition of G protein–coupled signaling has been shown to be profoundly time-dependent. Therefore, we studied the effects of long-term exposure to antidepressants on LPA signaling in oocytes.

Amitriptyline and Nortriptyline. Amitriptyline at 10^{-7} M, which did not affect LPA responses after short-term incubation, inhibited LPA responses significantly after a 2-h incubation (to 77% of the control response) without inducing membrane damage (fig. 2A). Similarly, a 120-min incubation in 10^{-5} M amitriptyline inhibited LPA responses to 71% of the control response (fig. 2A). Similar results were obtained after a 240-min incubation in amitriptyline at 10^{-7} M (to 59% of the control response) or 10^{-5} M (to 60% of the control response). Amitriptyline, even at high concentrations (10^{-4} or 10^{-3} M), did not affect cell integrity after long-term incubation. Similarly, nortriptyline did not interfere with membrane integrity, but at the greatest concentration tested (10^{-4} M), it blocked LPA signaling even more effectively than did amitriptyline (to 12% and 4% of the control response after 2 and 4 h, respectively).

Imipramine and Fluoxetine. Because high concentrations of these compounds were toxic to cells even after a 30-min exposure, only low concentrations were tested for prolonged incubation. Incubation for 120 min with imipramine (10^{-5} M) or fluoxetine (10^{-6} M) did not affect LPA-induced Cl currents in *X. laevis* oocytes (imipramine, 86 ± 42% of the control response; fluoxetine, 68 ± 32% of the control response; fig. 2B). Long-term exposure to these concentrations had a minimal effect on cell viability: after a 120-min exposure to antidepressants, none of the imipramine-treated cells and 20% of the fluoxetine-treated cells showed membrane damage (fig. 2B).

Effects of Nortriptyline on the Ca-activated Cl Channel

Inhibitory effects of the TCA could potentially be due to a direct effect on the endogenous Ca-activated Cl channel, which we used as a reporter for intracellular Ca release. Because this channel is specific to oocyte physiology, an action on this channel only would make the observed effects of TCAs of less interest to human medicine. Therefore, we determined the effect of a TCA on

Fig. 1. Effects of 30-min incubation in antidepressants on lysophosphatidic acid (LPA) signaling and membrane integrity in *Xenopus* oocytes. (*A*) Example trace of LPA (10^{-4} M)–induced peak Cl current under control conditions (*left*) and after a 30-min incubation in nortriptyline at 10^{-4} M (*right*). Effects of a 30-min preincubation in (*B*) nortriptyline (10^{-7}–10^{-4} M) or amitriptyline (10^{-7}–10^{-3} M) or (*C*) imipramine (10^{-7}–10^{-3} M) or fluoxetine (10^{-7}–10^{-4} M) on LPA (10^{-4} M)–stimulated Ca-activated peak Cl current (percentage of control response [*bottom*]) and loss of cell integrity (holding current, > 1 μA; expressed as percentage above incidence in control cells [*top*]). #Not determined. Data are mean ± SD. *$P < 0.05$ *versus* control.

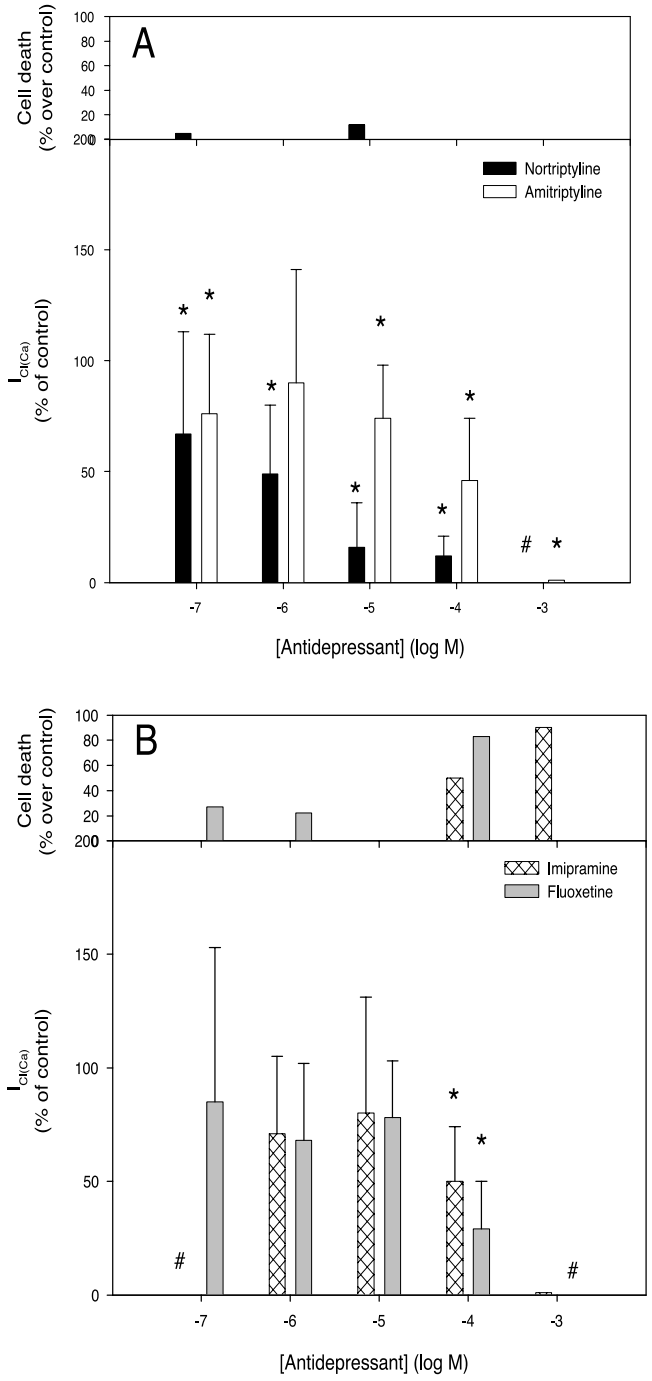


Fig. 2. Effects of a 120-min incubation in antidepressants on lysophosphatidic acid (LPA) signaling and membrane integrity in *Xenopus* oocytes. Effects of a 120-min preincubation in (*A*) nortriptyline (10^{-7}–10^{-4} M) or amitriptyline (10^{-7}–10^{-3} M) or (*B*) imipramine (10^{-6}–10^{-3} M) or fluoxetine (10^{-7}–10^{-4} M) on LPA (10^{-4} M)–stimulated Ca-activated peak Cl current (percentage of control response [*top*]) and loss of cell integrity (holding current, > 1 μA; expressed as percentage above incidence in control cells [*bottom*]). #Not determined. Data are mean ± SD. **P* < 0.05 *versus* control.

the oocyte Cl channel, by activating it directly using intracellularly injected $CaCl_2$. We studied nortriptyline in this experiment, because it showed the most pronounced inhibitory action on LPA signaling. Oocytes were incubated in nortriptyline at 10^{-5} M for 2 h, which resulted in greater than 80% inhibition of LPA signaling (fig. 2A), or in the vehicle as a control. Direct Cl channel activation was obtained by microinjection of 30 nl $CaCl_2$ (estimated final concentration, 10^{-5} M) into the oocytes under voltage clamp. Average currents induced in the presence of nortriptyline at 10^{-5} M were not different from the currents induced in the absence of the TCA ($P = 0.27$; fig. 3A); therefore, we conclude that the inhibitory effect of nortriptyline on LPA signaling cannot be explained by an action on the Cl channel.

Role of PKC

Alternatively, the inhibitory action of amitriptyline or nortriptyline on LPA signaling could be explained by an indirect effect. LPA signaling in oocytes is modulated by PKC[13]: PKC activation inhibits LPA responses, and PKC inhibition enhances LPA signaling. Hence, it is conceivable that the TCA might inhibit LPA signaling by activating PKC. To test this hypothesis, we determined the effect of nortriptyline on LPA signaling in the presence of the PKC antagonist inhibitor chelerythrine. Chelerythrine (estimated final concentration, 5×10^{-5} M) or a similar volume (30 nl) of KCl at 150 mM was injected into oocytes 2 h before experimentation. At the same time, the cells were incubated in nortriptyline at 10^{-5} M or in the vehicle as a control. In the absence of nortriptyline, chelerythrine enhanced LPA signaling, which is in agreement with our previous data[13] and confirms that the compound effectively inhibited PKC. Fractional inhibition of LPA-induced peak currents by nortriptyline was unaffected by the presence of the PKC antagonist (72% inhibition in control cells and 69% inhibition in the presence of chelerythrine; $P = 0.41$; fig. 3B). Thus, inhibition of LPA signaling by nortriptyline appears not to depend on PKC.

Discussion

Our data show that the structurally closely related TCAs nortriptyline and amitriptyline, when applied for several hours at clinically relevant concentrations, inhibit LPA signaling in *Xenopus* oocytes. These effects cannot be attributed to either a direct action on the oocyte Ca-activated Cl channel or activation of PKC. In contrast, the TCA imipramine and the specific serotonin reuptake inhibitor fluoxetine, at these concentrations, do not inhibit LPA signaling. However, at greater concentrations, these compounds compromise membrane integrity, an effect that may be of relevance if these TCAs are considered for use as long acting, locally injected analgesics.

LPA

As mentioned previously, we chose the LPA receptor for study primarily because the interactions of LAs with

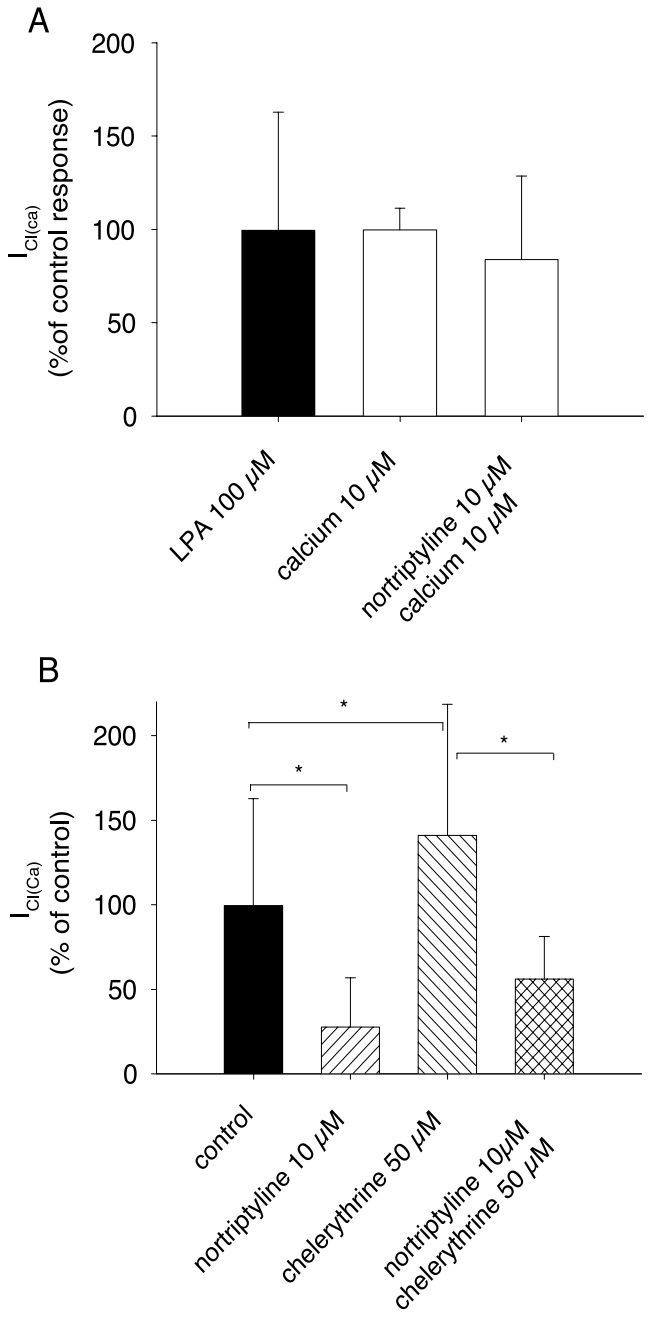


Fig. 3. (*A*) Nortriptyline has no effect on Ca-activated Cl channels. Intracellular injection of $CaCl_2$ (final concentration, 10 μM; *second bar*) induced Cl currents similar to those induced by lysophosphatidic acid (LPA) (100 μM; *black bar*). Nortriptyline (10 μM) did not affect Ca-induced Cl currents (*third bar*; percentage of control). Data are mean ± SD. (*B*) Effects of protein kinase C (PKC) on LPA signaling. PKC inhibition with chelerythrine (50 μM) did not affect the inhibitory effect of nortriptyline (10 μM) on LPA (100 μM)–induced Cl currents (percentage of control). Data are mean ± SD. *$P < 0.05$.

its signaling pathway have been investigated in some detail. In addition, the compound may potentially be relevant to inflammatory pain. LPA is an intercellular phospholipid mediator known to induce a variety of biologic responses (*e.g.*, cell proliferation, platelet aggregation, smooth muscle cell contraction, chemotaxis, and inhibition of differentiation).[22] As an inflammatory mediator, it affects migration and metabolic activity of neutrophils.[14] LPA can be generated by platelets, leukocytes, and other cells stimulated with inflammatory agents[23] and activates several specific subtypes of membrane-bound G protein–coupled receptors in a variety of cells, thereby activating intracellular signaling cascades. In mice, LPA enhances wound healing.[18] *In vivo* studies demonstrated that LPA enhances nociceptive activity on nociceptor endings of primary afferent neurons and that LPA receptors are involved in peripheral pain transmission.[19,20]

Analgesic Actions of TCAs

The mechanisms by which TCAs are antinociceptive remain unclear. Interactions with signaling of biogenic amines,[24] adenosine,[25] opioids,[26] serotonin, norepinephrine, histamine, and acetylcholine,[27–29] as well as *N*-methyl-D-aspartate receptors[30] and ion channels,[31] have been reported. Whereas amitriptyline has been widely used and has demonstrated analgesic properties,[2,3] fluoxetine has limited efficacy.[1] Furthermore, peripherally administered fluoxetine in rats caused edema by accumulation of serotonin.[32] It has been hypothesized that the limited analgesic efficacy of fluoxetine might result from the pronociceptive effect of serotonin counteracting the antinociceptive properties of the specific serotonin reuptake inhibitor. In our study, we also observed different effects of fluoxetine and amitriptyline: fluoxetine damaged cells, but in undamaged cells, it had no effect on LPA signaling; in contrast, amitriptyline did not compromise membrane integrity but inhibited LPA signaling. Thus, an alternative hypothesis for the differential clinical efficacy of these compounds might be their different actions on G protein–coupled signaling. If the effects of TCAs on G protein–coupled receptor signaling are related to their analgesic properties, we would predict that nortriptyline might be even more effective as an analgesic than amitriptyline, whereas imipramine would not be effective.

Comparison of LAs and TCAs

The effects of amitriptyline and nortriptyline on LPA signaling resemble those of LAs (although TCAs seem to be 10- to 100-fold more potent than LAs), but the effects of imipramine and fluoxetine are quite different. The different effects of amitriptyline and imipramine are remarkable, considering their structural similarities. LAs, at low concentrations applied for long periods, block LPA signaling in *Xenopus* oocytes profoundly but do not affect cell integrity even at high millimolar concentrations.[12] Although the actions of amitriptyline or nortriptyline on LPA signaling superficially resemble those of LAs, it cannot be assumed that they act through the same mechanism. Similar to the observations reported here with nortriptyline, we previously observed that LAs in-

hibit LPA signaling without affecting either the Ca channel or PKC.[13] Nonetheless, we do not know if the actions of amitriptyline and nortriptyline are mediated by interactions with the same molecular target identified for LAs (*i.e.*, the Gq protein). Regulation of receptor expression should be considered as an alternative mechanism of action. Desipramine, administered intraperitoneally in rats, activated G protein receptor kinases within several hours, but fluoxetine did not.[33] These effects might, however, be indirect, because desipramine, but not fluoxetine, increases norepinephrine concentrations in the brain.

Limitations of the Model

It should be kept in mind that ours is a single-cell *in vitro* model, and our data should not be extrapolated to the clinical setting. Several caveats should be considered. First, we studied amphibian rather than mammalian receptors. However, the LPA receptor in oocytes has been shown to function similar to its mammalian counterpart.[22] Second, we studied only a single form of LPA signaling (Ca-induced Cl currents), whereas several intracellular signaling cascades are activated by LPA (including modulation of cyclic adenosine monophosphate concentrations through Gi). It is possible that these other actions might be affected differently by TCAs. Indeed, we recently studied TCA effects on human neutrophils and obtained results that were, in some respects, different from the data obtained for oocytes. We observed no TCA effect on platelet-activating factor–induced neutrophil priming (in contrast to an inhibiting effect of LAs[34]), and all TCAs tested in the current study induced a significant degree of toxicity in neutrophils.[35] Several issues may account for this difference, but most important is probably that we studied different receptors in these models: platelet-activating factor in neutrophils and LPA in oocytes. However, in both models, we observed a degree of toxicity induced by at least some of the compounds at concentrations used when TCAs are injected as long-acting anesthetics.

Cellular Toxicity of TCAs

Toxic actions of antidepressants—in concentrations used in this study—have been observed previously by others in cultured rat C6 glioma and human astrocytoma cell lines.[36] However, these effects have not been considered clinically relevant, because such concentrations are not attained during routine clinical use. Therapeutic blood concentrations of imipramine or fluoxetine for treatment of depression are $0.5–1 \times 10^{-6}$ M. For use in chronic pain states, therapeutic concentrations are even lower than required for antidepressant effects.[37] However, this assessment of the relevance of cytotoxic action changes when these compounds are to be used as LAs and injected locally. Under these conditions, concentrations of up to 10^{-2} M are used,[5,6] which are 10-fold greater than the maximally toxic concentration observed in the current study. Thus, their cellular toxicity could become relevant in that setting. Incubation of HL-60 cells in 8×10^{-5} M imipramine induced loss of mitochondrial membrane potential and apoptotic DNA fragmentation in a time-dependent manner.[38] TCAs have therefore even been considered as cancer chemotherapeutics. The effects observed in the current study thus fit a clear pattern, which should be considered because these compounds are being developed for use as LAs.

Summary

We have shown that nortriptyline and amitriptyline, TCAs with structural similarity to LAs and with several documented LA properties, block LPA signaling in *X. laevis* oocytes with potencies 10 to 100 times greater than LAs. In contrast, the TCA imipramine and the structurally unrelated selective serotonin release inhibitor fluoxetine do not block LPA signaling but are toxic when applied at concentrations that would be attained routinely if the compound were used for local anesthesia, as is being investigated for amitriptyline. These findings might explain, in part, the analgesic efficacy of systemically administered TCAs in chronic pain states and raise some concerns about local administration of such compounds in high concentrations.

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