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Nicotinic Antagonists (Piperidines and Quinuclidines) Reduce the Susceptibility of Early Sea Urchin Embryos to Agents Evoking Calcium Shock

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ABSTRACT. 1. Some nicotinic antagonists (piperidine and quinuclidine derivatives and bis-quaternary compounds) protect early embryos of the sea urchin *Lytechinus pictus* against a calcium shock evoked by ionomycin or a mixture of phorbol myristate acetate and nicotine.

2. Maximal protective potency was found for drugs that did not penetrate the plasma membrane.

3. Early sea urchin embryos have nicotinic acetylcholine receptors (nAChR) or nAChR-like structures localized on the cell surface that, apparently, take part in the control of Ca^{2+} influx. GEN PHARMAC 29;1:49–53, 1997. **© 1997 Elsevier Science Inc.**

KEY WORDS. Early embryos, nicotinic choline receptors

INTRODUCTION

Acetylcholine (ACh), like other classical neurotransmitters (serotonin and the catecholamines), is a multifunctional regulator functioning throughout all development (Buznikov, 1990, 1991; Buznikov *et al.*, 1996). In particular, it functions as a trigger and regulator of the cleavage divisions that immediately follow fertilization in all animals studied, including sea urchins and amphibians (Buznikov, 1990). It was found in early sea urchin embryos that ACh acts through two groups of cholinoreceptors (or, more cautiously, functional receptor analogs). The first group seems to be quite unusual when compared with known cholinoreceptors of differentiated cells and is located entirely intracellularly (Buznikov, 1984, 1990). The second group is very similar to the nicotinic cholinoreceptors (nAChRs) of adult vertebrates (Falugi, 1993). Preliminary data suggest but do not definitely prove that the nAChRs of early embryos are located on the cell surface and take part in the control of Ca^{2+} influx and the maintenance of cytoplasmic Ca^{2+} homeostasis (Buznikov, Shmukler and Whitaker, unpublished).

It seemed likely that an experimental model that we described earlier (Buznikov *et al.*, 1993a) might allow us to test this idea. We found that ionomycin (about 0.5–1.0 μM) or a mixture of phorbol 12-myristate 13-acetate (PMA; >0.02 μM) and nicotine (>10–40 μM) causes lysis of early sea urchin embryos 10–15 or 30–60 min, respectively, after addition to the artificial sea water (ASW) bathing the embryos. The increase of intracellular Ca^{2+} concentration to lethal level (Ca^{2+} shock) seems to be the direct cause of the effect; the calcium increase is evoked by Ca^{2+} influx from ASW through the plasma membrane. Nicotine itself used at concentrations as high as 200 μM does not affect cleavage divisions. PMA (0.02–100.0 μM) itself has a cytostatic effect and evokes very typical anomalies (the so-called PMA syndrome) but does not kill the embryos within 6–12 hr after addition to ASW (Buznikov *et al.*, 1993a).

To pharmacologically examine the role of nAChR on the regulatory machinery of early embryogenesis, we used the aforementioned model treated with a series of piperidine and quinuclidine derivatives that are known as neuronal nicotinic antagonists (Barlow, 1964; Papke *et al.*, 1994; Triggle and Triggle, 1976) and act on the open state of nAChR ion channels (Lingle, 1983; McDonald *et al.*, 1995; Van Rossum, 1962).

MATERIAL AND METHODS

In all experiments, early embryos of sea urchin *Lytechinus pictus* were used, obtained and handled in accord with standard procedures (Buznikov and Podmarev, 1990). Each experiment was conducted on the fertilized eggs of one female; therefore, the number of experiments in each series was the same as the number of females used. Artificial seawater (ASW) was used as incubation medium and solvent. The substances being tested were added to the medium 15 min after fertilization. The results were evaluated visually and recorded by means of microphotography. Commercial preparations (RFR) of neuronal nicotinic antagonists imechine (I), temechine (II), pempidine (III), tempidone [syn. triacetonamine, vincubine (V)], and nanofine (VI) were used. Compound IV (LK-58 available as tempetol from Latoxan, France) was synthesized by a known procedure (Nikitskaya *et al.*, 1974). The formulas of quinuclidine (I and II) and piperidine (III–VI) antagonists are shown in Fig. 1. Bis-quaternary compounds [the neuronal nicotinic agonist subecholine (RFR commercial preparation) and antagonist hexamethonium and the muscle nicotinic antagonist *d*-tubocurarine (Sigma, USA)] were used for comparison. The nicotinic agonist nicotine sulfate, the protein kinase C activator phorbol 12-myristate 13-acetate (PMA) and the Ca-ionophore ionomycin also were from Sigma (USA).

Some peculiarities of the neuronal nicotinolytics that we used should be noted. Owing to a pK^a>11 (Hall, 1957), more than

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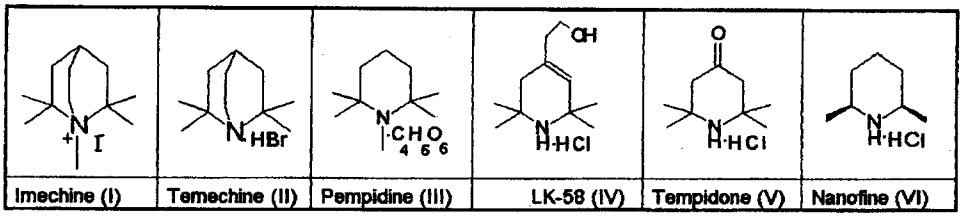

FIGURE 1. The structural formulas of piperidine and quinuclidine derivatives tested: I, imechine; II, temechine; III, pempidine; IV, LK-58 (tempetol); V, tempetone (vincubine); VI, nanophine.

99.98% of compounds II, III and VI at pH 7.4 exist in a biologically active cationic form but may readily permeate biological membranes in their minor neutral form, in contrast with the permanently charged bis-quaternary compounds (subecholine, hexamethonium and *d*-tubocurarine). The latter do not permeate plasma membranes and do not enter the cytoplasm [at least, in early sea urchin embryos—see Buznikov (1984, 1990)]. The less-basic compound, V (pK_a 7.80), showed decreased peripheral antinicotinic potency in animal tests (only 71.5% is in the protonated form at pH 7.4) but enhanced sedative properties (owing to increased brain-blood–barrier permeation; Chodera *et al.* (1964)]. The conversion of "hot" peroxy radicals into stable TEMPO radicals (a characteristic of 2,2,6,6-tetramethylpiperidines) can occur in V (Rozantsev *et al.*, 1992).

RESULTS

In the first experimental series (five experiments), it was found that none of the nicotinic antagonists and agonists tested affected the early cleavage divisions at concentrations as high as 200 μM. In the second (seven experiments) and third (seven experiments) series, we attempted to evaluate the experimental effects of nicotinic antagonists on the sensitivity of early embryos to lethal doses of ionomycin or the mixture of PMA and nicotine. The data obtained in these two experimental series and shown in Table 1 and Fig. 2 are quite similar. It was found that all the nicotinic antagonists tested and subecholine have some protective action against both ionomycin and PMA+nicotine. Maximally protective action was found for compounds I, II and IV and for the bis-quaternary drugs hexamethonium and *d*-tubocurarine.

It was found that sea urchin embryos were completely insensitive to minimum lethal doses of ionomycin (0.5–1.0 μM) in the presence of compounds I, II or IV (20–50 μM). Sometimes compounds I and IV were more active in these experiments in comparison with compound II. In these cases, the proportion of embryos fully protected from the action of twice the lethal dose of ionomycin (1.0–2.0 μM) was lower for compound II and higher for compounds I and IV. The protective actions of compounds III, V and VI, as well as subecholine, were less pronounced. The proportion of protected embryos with these last compounds was always lower than 100%, and they were protected only partially (even at threshold concentrations of ionomycin). Compounds III and V and subecholine had almost equal protective activity. The protective action of compound VI was the lowest and sometimes near zero.

In the experiments with PMA+nicotine, embryos protected by compounds I, II or IV showed only the PMA syndrome and remained alive as long as the embryos treated with PMA alone. Maximal protective action was found for compound I (imechine), *d*-tubocurarine and hexamethonium, which all sometimes partially reversed the PMA syndrome; that is, partially normalized the development of embryos (Fig. 2). The activity of the other compounds was much lower. Compound III (pempidine) prolonged the life of embryos but did not weaken the potentiation of the PMA effect by nicotine (Fig. 2). As in experiments with ionomycin, the protective action of compound VI (nanofine) was the lowest.

DISCUSSION

There are good reasons to believe that the protective action of nicotinolytics and subecholine against the lethal effects of ionomycin or PMA+nicotine is caused by prevention of Ca^{2+} influx from the ASW to the cytoplasm of embryos. Such a conclusion is supported by the preliminary observations that the lytic effects of ionomycin and PMA+nicotine are absent in calcium-free ASW and by our preliminary experiments with direct measurements of Ca^{2+} in the cytoplasm of early sea urchin embryos. These pharmacological effects appear to show the presence of NAChR-like receptors in early *L. pictus* embryos as well as participation of these receptors in the control of the cytoplasmic Ca^{2+} level. However, it is unclear how NAChR-like receptors can modulate ionomycin-induced calcium influx, because there are no precedents for modulation of ionophore-mediated calcium transport. Nonetheless, the results that we obtained provide some additional information about the role, localization and peculiarities of these receptors. Before discussing this

TABLE 1. The effect of nicotinic antagonists and subecholine on the sensitivity of one-cell embryos of *Lytechinus pictus* to lethal doses of ionomycin (1.0 μM) and PMA (0.05 μM) + nicotine (40 μM)

	I	II	III	IV	V	VI	Tubo	Sube	Hexa
Ionomycin	100*	80–100	40–60	100*	30–50	0–10	100*	75–100*	100*
PMA + nic.	100*	100	40	100*	20–35	0–5	100*	100*	100*

Note: The percentages of living embryos are shown. These results were obtained 3 hr after the death of all the embryos treated by ionomycin or the mixture PMA + nicotine alone. All the nicotinergic substances were used at concentrations of 20 μM. Abbreviations: tubo, *d*-tubocurarine; sube, subecholine; hexa, hexamethonium.

* These were the embryos with fully or partly normalized development.

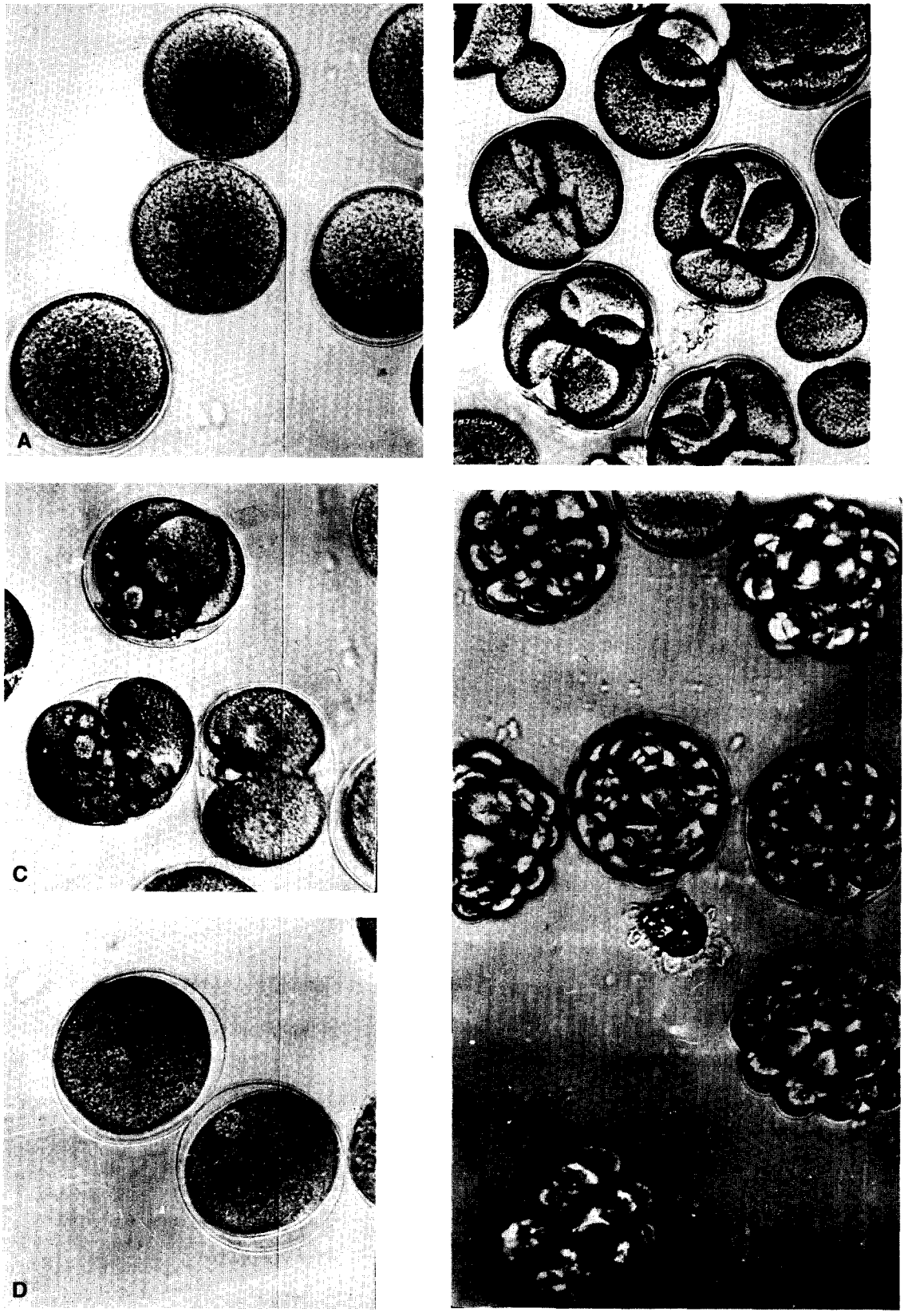

FIGURE 2. The protective action of nicotinic antagonists against the lethal effects of a mixture of PMA (0.05 μM)+ nicotine (20 μM) (experiment on *Lytechinus pictus* embryos; all the substances were added 15 min after fertilization). (A) PMA+nicotine alone; death and lysis of embryos. (B) The same as (A)+imechine (20 μM); all the embryos were alive and cleaving. (C) The same as (A)+temechine (20 μM); all the embryos were alive but blocked on the two-cell stage; the PMA syndrome (cytostatic effect, extrusion of small cytoplasts near the cleavage furrow, giant polyploid cell nuclei, etc.) was clearly evident. (D) The same as (A)+pempidine (20 μM); many embryos were alive but did not cleave; PMA syndrome was potentiated by nicotine. (E) Control embryos (early blastula stage).

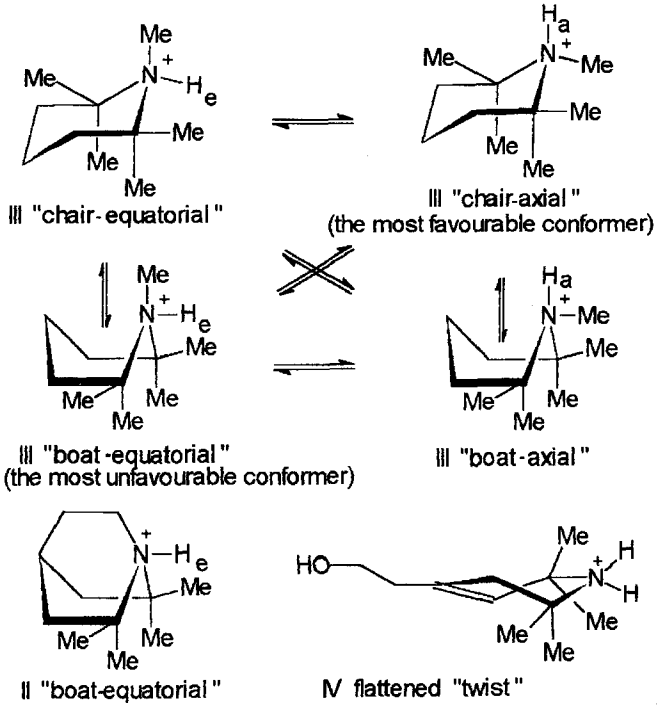

FIGURE 3. Conformational details for compounds II, III and IV.

further, it is necessary to point out several specific structural features of the compounds tested.

Pairs of indole derivatives (tertiary amines with corresponding methiodide analogs) were used successfully to study 5-HT receptor localization in oocytes and early embryos of echinoderms and amphibians (Buznikov *et al.*, 1993b). The protective action of the nonpermeant bis-quaternary compounds (*d*-tubocurarine, hexamethonium and subecholine) against ionomycin or PMA+nicotine suggests that the nAChRs are localized on the cell surface.

However, the 1,2,2,6,6-pentamethylpiperidines are not so straightforward, because their quaternary salts decompose under physiological conditions (Frachey *et al.*, 1993). Quaternary quinuclidinium salt I is stable and is structurally very similar to the parent amine II; but, owing to its unusual fixed "boat" conformation with all three piperidine rings incorporated into a rigid bicyclo [2.2.2] octane cage, its $^{+}$N–H or $^{+}$N–CH3 bonds are oriented strictly equatorially (Fig. 3). In contrast, owing to an easy "chair-boat" interconversion and nitrogen inversion (equatorial or axial position of the $^{+}$N–H group), piperidines exist as a mixture of four conformers: chair-axial, chair-equatorial, boat-equatorial, and boat-axial. The requirement of minimal steric repulsion of the bulky methyl groups makes the axial $^{+}$N–H form predominant (99%) (Navajas *et al.*, 1990). Thus, to interpret our pharmacological results precisely, we needed to use a minimal set of three compounds (I–III) and compare the pair of quaternary/tertiary quinuclidines (I/II) to determine nAChR localization and the pair of tertiary bi- and monocyclic analogs II/III to exclude the possibility of a structural difference that might affect the ligand-receptor interaction. The three further secondary analogs of compound III allow us to estimate the role of hydrophobicity (IV), basicity (V), and degree of 2.6-substitution on nicotinic antagonism of piperidines.

The high and approximately equal potency of I, II and bis-quaternary nicotinolytics in suppressing Ca^{2+} shock (Table 1) implies the absence of a lipophilic membrane permeation step for the action of these compounds; that is, it demonstrates unequivocally the localization of nAChR receptors to the surface of embryo cells. A slightly higher activity of quaternary salt I compared with II was found in some experiments. This is interesting, because it shows the prevailing effect of an increase in hydrophobicity close to the cationic nitrogen over the increase in its steric shielding. The lower activity of compound VI compared with III is in accordance with the results obtained in somatic tissues (Brehteric *et al.*, 1959).

All the results, including the demonstration of specific action of nicotine on the PMA-treated embryos, suggest that these receptors are members of the nAChR family. However, the considerably lower potency of compound III compared with II reveals the importance of $^{+}$N–H orientation, which is not characteristic of any known group of nAChRs (Brehteric *et al.*, 1959; Mashkovsky *et al.*, 1983). This phenomenon is not simply due to a difference in basicity, because compounds II, III and VI are effectively completely protonated (Hall, 1957). There are other data demonstrating the uniqueness of nAChR in early sea urchin embryos. Subecholine acts as nicotine antagonist on these embryos (as described earlier) but as a nicotinic agonist on all somatic cells studied, including sea urchin muscle (Michelson, 1973). Moreover, the antagonists of neuronal (hexamethonium and compounds I, II and IV) and muscle (*d*-tubocurarine) nAChR had virtually equal protective activities in our experiments. One cannot exclude the possibility that both neuronal and muscular types of nAChR are present on the plasma membrane of early sea urchin embryos, but it is very unlikely. It is more probable that these embryos have a unique nAChR (perhaps the ontogenetic precursors of adult nAChR comparable to embryonic nAChR in vertebrates). It would be interesting to follow up this suggestion by using radioligand and molecular cloning techniques.

The high protective potency of compound IV may be caused by the flattening of the piperidine ring due to the introduction of the endocyclic double bond (fixed flattened "twist" conformation) or to the presence of a hydroxyethyl "tail." No comparable data have been obtained in adult animals, and this interesting problem must be investigated separately.

It is obvious that the nAChR under consideration takes part in the influx of Ca^{2+} from the external medium (ASW in this case) in modulating the cytoplasm—that is in controlling cytoplasmic Ca^{2+} transients after fertilization. These receptors may be functionally coupled to Ca channels found in the plasma membranes of early sea urchin embryos (Yazaki *et al.*, 1995). It is also possible that the receptor-operated ionic channels of nAChR mediate Ca influx in the preceding experiments. If so, the protective effect of nAChR antagonists and subecholine could be caused by their direct blocking action on these ionic channels. Apparently, this mechanism of action is different from that of bis-quaternary compounds or piperidine and quinuclidine derivatives (Gilmore *et al.*, 1995; McDonald *et al.*, 1995).

Imechine, hexamethuonium and *d*-tubocurarine weakened, whereas ACh and nicotine potentiated, the action of PMA on early *L. pictus* embryos (see Fig. 2). Consequently, the embryonic nAChR of sea urchins may also be functionally coupled to the protein kinase C pathway. ACh is the most probable endogenous ligand for these receptors.

Thus, the results obtained show a possible functional coupling of prenervous ACh to the phosphoinositide-diacylglycerol-calcium signaling system in early development and provide an adequate experimental model for the study of this coupling. A comparison of the biological activities of the imechine-temechine-pempidine triad and other related drugs has allowed us to use this model not only to

prove the surface membrane localization of embryonic nAChR, but also to study the interaction of these receptors with specific ligands.

As for the function of prenervous ACh as a possible trigger and regulator of cleavage divisions, it seems that embryonic nAChRs do not take part in cell-cycle control directly. In fact, neither nicotine nor the nAChR blockers used have direct effects on the cleavage divisions of *L. pictus*. In addition, all cytostatic ACh antagonists seem to act at the intracellular level (Buznikov, 1984, 1990). At the same time, embryonic nAChR may participate in the control of cleavage divisions indirectly, through the regulation of the cytoplasmic Ca^{2+} transients and protein kinase C activity.

A practical result of this work is the discovery of a series of structurally related compounds with considerably higher calcium-blocking potency than that described earlier for compound V (Xu *et al.*, 1988). Experiments with other piperidine and quinuclidine nicotinic antagonists are now in progress.

CONCLUSION

Pharmacological data indicate that early (one-cell or cleaving) embryos of the sea urchin *L. pictus* have nAChRs on the cell surface. Prenervous ACh action appears to be functionally coupled with the phosphoinositide-diacylglycerol-calcium signaling system of early embryos through these receptors.

SUMMARY

Some nicotinic antagonists (piperidine and quinuclidine derivatives and bis-quaternary compounds—*d*-tubocurarine, hexamethonium) and the agonist subecholine used at concentrations of 20–50 μM protect early embryos of the sea urchin *L. pictus* against a calcium shock evoked by ionomycin (0.5–1.0 μM) or a mixture of PMA (>0.02 μM) and nicotine (10–40 μM). Maximal protective potency was found for drugs in the medium that did not permeate into the cytoplasm. Early sea urchin embryos, therefore, have nicotinic acetylcholine receptors (nAChR) or nAChR-like structures localized on the cell surface that can apparently modulate Ca^{2+} influx. Judging from the virtually equal protective potencies of antagonists of neuronal (hexamethonium, imechine and temechine) and muscular (*d*-tubocurarine) nicotinic receptors and from the nicotinolytic activity of subecholine, these embryonic nAChRs have pharmacological peculiarities that have not been described for any nAChR in differentiated cells.

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