EFFECT OF CALCIUM CHANNEL ANTAGONISTS AND OTHER AGENTS ON OLFACTORY RECEPTION

THESIS

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By

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The role of Ca^{++} in olfactory responses was investigated with inorganic and organic calcium channel antagonists. Electrophysiological responses to odorants were recorded from frog olfactory mucosa before and after aerosol application of different agents. Electroolfactogram responses were blocked by certain inorganic ions with the order of effectiveness $Zn^{++}>Ln^{+++}>Cd^{++}>Ca^{++}>Co^{++}>Sr^{++}>Mg^{++}$. Ba^{++} potentiated olfactory responses, and is known to potentiate calcium channel-mediated responses in other tissues. Certain local anesthetics which are thought to act through calcium channel blockade were inhibitory to olfactory responses, with the order of effectiveness being dibucaine>tetracaine>procaine. These data support the idea that Ca^{++} is involved in olfaction, perhaps acting as a current carrier and/or a second messenger.

Preliminary experiments on channel localization were performed using a silicon-labeled amine. Attempts to localize the silicon label were inconclusive, although silicon was detected in the olfactory tissue.

TABLE OF CONTENTS

LIST OF TABLES

Table

Page

LIST OF ILLUSTRATIONS

Figure

CHAPTER I

INTRODUCTION

Despite a number of studies on the initial events of olfactory reception, relatively little is known of the detailed nature of the receptor sites associated with olfactory receptor neurons. In addition, the nature of ion channels of the receptive membranes, the identity of the ions involved in transduction, and participation of second messenger systems in olfactory transduction have yet to be elucidated.

Olfactory Receptor Morphology

In all vertebrates, olfactory receptor cells are scattered among supporting cells in the olfactory mucosa. The olfactory receptors are bipolar neurons, whose axons form the olfactory nerve. The cell body is located near the base of the epithelium and a single dendrite extends to the surface of the mucosa where it forms an expanded region called the olfactory knob (Yamamoto et al., 1965; Moulton and Beider, 1967). One to twenty cilia arise from each olfactory knob. Two types of cilia have been reported, one motile and 20-50 um in length, and the other nonmotile and up to 200 um in length (Reese, 1965; Mair et al., 1982). These may represent different developmental stages in the

life of the receptor neuron (Mair et al., 1982).

Freeze-fracture studies of many different types of cilia, ranging from bovine olfactory cilia (Menco et al., 1976) to mussel gill cilia (Gilula et al., 1972) to the cilia of Paramecium (Dute and Chung, 1976) have disclosed a ring of intramembraneous particles, the "ciliary necklace", at the base of each cilium. Gilia et al. (1972) suggested that the necklace serves as the site of ion channels which can alter membrane permeability and affect motility. Moran et al. (1977) proposed that the ciliary necklace participates in mechnoelectric transduction in a sense organ, the grasshopper proximal chordotonal organ. Dunlap and Eckart (1984) have shown that calcium channels in Paramecium are located on the ciliary membrane near the axial portion, an area which corresponds to the ciliary necklace.

Morphological identification of olfactory receptor sites has been attempted using freeze-fracture techniques on olfactory mucosae of various organisms (Menco et al., 1976; Usukura and Yamada, 1978; Menco, 1980; Mirgall, 1983). Usukura and Yamada (1978) showed the presence of particles 11 nm in diameter on the cilia of olfactory neurons of the newt, Cynops pyrrhogaster. These particles were absent from respiratory epithelium. Menco et al. (1976) described membrane particles ranging in sizes from 8-12 nm in diameter on bovine olfactory cilia.

Biochemistry of Olfactory Receptors

The molecular sites where odorants interact with olfactory neurons are generally thought to be membrane receptors. These receptor sites are probably proteinaceous, because protein specific reagents modify behavioral and physiological responses to odors and interfere with the binding of odorants to fractions of olfactory tissue (Price, 1978; Rhein and Cagan, 1981, 1984; Kleene and Gesteland, 1981; Fesenko et al., 1983; Mason et al., 1984; Schafer et.al., 1984a, 1984b). The receptor molecules are probably located on the bases of the cilia of the olfactory receptor neurons, although the only data to support this supposition are measurements of electrophysiological latencies, rather than morphological or biochemical data (Getchell et al., 1981). There is a consensus of workers in the field that the most carefully done study is that of Cagan (1980) who measured binding of radioactively labeled odorants (amino acids) to a receptor-enriched medium extracted from the olfactory organs of salmonid fishes. Cagan's studies indicated that the binding sites are proteinaceous and associated with ciliary membranes, since the highest binding levels were demonstrated in an isolated ciliary fraction.

Chen and Lancet (1983) performed biochemical assays of isolated cilia of amphibians and compared protein profiles of olfactory (sensory) cilia to that of respiratory (non-sensory) cilia, reasoning that proteins unique to sensory cilia might be receptor proteins or other proteins

associated with the transduction process. They showed that olfactory cilia have four seperate membrane-bound glycoproteins which are absent in respiratory cilia (Chen and Lancet, 1983). One of these proteins is a "G-protein" which binds GTP and in other tissues is involved in cyclic AMP-mediated second messenger actions.

Possible Roles of Ca^{++} in Olfaction

Ca^{++} plays important roles in many cellular events, such as neurotransmitter release, muscle contraction, and the regulation of membrane ion permeability (Glossman et al., 1982; Reuter, 1983; Synder, 1984). Not surprisingly, calcium-dependent second messenger systems have been proposed to be a part of the olfactory transduction mechanism. Menevse et al. (1977) examined the effects of phosphodiesterase inhibitors and dibutryl cAMP on the olfactory potentials of the frog, Rana temporaria. Both the phosphodiesterase inhibitors and dibutryl cAMP decreased electrophysiological responses (electroolfactograms or EOG's) from olfactory receptor neurons. These findings may also implicate Ca^{++} in olfactory transduction, for Reuter (1983) has suggested that dibutyryl cAMP and phosphodiesterase inhibitors modulate calcium channels through phosphorylation of membrane proteins associated with the channels.

Local anesthetics have also been proposed as calcium

channel modulators. Popahadjopoulos (1971) and Low et al. (1978) have suggested that local anesthetics disrupt or displace Ca^{++} by dissolving in the phospholipid of the cell membrane and increasing the distance separating components of the Ca^{++} attachment site. Volpi et al. (1981) have proposed that local anesthetics also act by antagonizing calmodulin, the calcium-binding protein. They suppose that local anesthetics interefer with normal function of the calcium-calmodulin complex which regulates phosphodiesterase and protein kinases involved in second messenger systems.

Inorganic ions which compete with or replace Ca^{++} can alter physiological function of calcium-activated processes. For example, barnacle muscle fiber potentials are mediated by voltage-gated calcium channels (Hagiwara and Takahashi, 1967). These investigators examined the effects on the action potential when a variety of divalent and trivalent cations were applied. Ions such as Ln^{+++}, Zn^{++}, and others had an antagonistic effect on the calcium-mediated action potential. Typically, the order of effectiveness of inorganic ions in inhibiting calcium channel-mediated functions is as follows: $La^{+++}>Zn^{++}>Co^{++}>Ca^{++}>Mg^{++}>Sr^{++}$.

Since olfactory receptors are probably located on cilia, and since some of the cilia are apparently motile, it is appropriate to consider the effects of ionorganic ions on ciliary motility. Natoh and Kanko (1972) modified ciliary activity in Paramecium by the addition of various divalent cations to the culture medium. They showed that different

cations elicit different beating patterns of the cilia. Ca^{++}, Ba^{++}, and Sr^{++} all induced reversal of the normal beating pattern, while Mg^{++} and Co^{++} reactivated the normal ciliary motion. They suggested that movement and synchronicity of the cilia are controlled by the concentration of free Ca^{++} present. Ciliary movement in olfactory receptor neurons has been proposed to be dependent on Ca^{++} concentrations. Mair et al. (1982) did studies on olfactory receptor cilia of the frog, Rana pipiens, and showed that Ca^{++} levels induced changes in the pattern of ciliary movement. Ciliary motility decreased, then stopped, in a calcium-free medium including the calcium chelators EGTA and EDTA. Addition of La^{+++} and Co^{++} in a medium containing normal Ca^{++} levels caused jerky, uncoordinated movements of the cilia. They proposed that the altered ciliary movements are "related to the after-effects of odorants on calcium ionophores" (Mair et al., 1982).

Local anesthetics have also been shown to affect ciliary movement in Paramecium. Browning and Nelson (1976) examined the effects of tetracaine and procaine on ciliary beating in Paramecium, and found that these local anesthetics stimulate ciliary reversal. They hypothesized that local anesthetics act by affecting calcium channels through interaction with the surrounding lipid bilayer, as also suggested by Papahadjopoulos (1971) and Low et al. (1978).

Preliminary evidence suggests that Ca^{++} plays a role in olfactory transduction. Suzuki (1978) examined the effects of various inorganic ions on olfactory receptor potentials in the lamprey, Entosphenus japonecus. Lamprey olfactory responses were suppresed by La^{+++} and Co^{++} ions, leading Suzuki (1978) to suggest Ca^{++} may play an important role in the generation of olfactory receptor potentials.

Statement of Problem

The purpose of this research was to study the possible role of Ca^{++} in olfactory transduction. Inorganic and organic Ca^{++} channel antagonists and chelators of divalent cations were used. The potential for using a silicon-labeled amine as an electron-dense marker of the receptor/ionophore complex was also explored.

CHAPTER II

MATERIALS AND METHODS

Stimulation

Odorous stimuli in the form of pure chemicals in the vapor phase saturated in air were delivered at 100 sec intervals and were always of 0.3 sec duration. In preliminary attempts to label olfactory receptors with a silicon-labeled amine ($(Ch_3)_3SiOCH_2NH_2$ or IMMT-9) exposures of 15 sec were used. Aerosol application of liquid reagents will be described later. Isoamyl acetate ($CH_3COOCH_2CH_2CH(CH_3)_2$, or IAA) was used as a reference odorant in all experiments because (i) IAA's adapating effects were rapidly and totally reversible even when using air saturated with the stimulant, (ii) ester-sensitive receptors have a more uniform distribution in the olfactory mucosa than most other types (Mackay-Sim and Kubie, 1981), making electrode placement less critical, and (iii) IAA or a related ester has been used by virtually all others who have experimented on the amphibian olfactory system. Experiments were only performed on animals which initially produced consistent electroolfactogram (EOG) responses to IAA with an amplitude of 1 mV or more.

A four channel stimulation and recording system was

used in this study (Figure 1). It is capable of automatically delivering any of four different odorants in a timed sequence, although only one odorant (IAA) was used in most experiments. Automatic stimulation was programmed using an eight-bank program timer (Lafayette model 52023), which drove a system of low current relays which actuated the system of electric valves in the olfactometer.

Stimulating air was passed through activated charcoal, humidified, then delivered to the olfactory mucosa. The delivery rate used was 950 ml/min through a 0.75 cm i.d. orifice situated 4.3 cm from the mucosa. The odorant reservoir contained sufficient odorant in liquid form (0.5 ml dispersed on a 5 cm disk of filter paper) to saturate a 1,000 ml air volume.

Inorganic ions and organic agents dissolved in water were applied in solution as an aerosol, with application initiated manually through a fifth stimulating channel driven by a seperate solenoid valve system controlled by a Grass S44 electronic stimulator. A glass no. 40 DeVilbiss nebulizer intended for drug inhalation was used to deliver aerosols to the olfactory mucosa. The nebulizer delivery tube was slightly modified to connect to a 0.75 cm i.d. barrel aimed at the olfactory mucosa 4.3 cm away. The rate of aerosol delivery was calculated to be 0.01 ul/sec, or for a 0.1 M solution loaded in the nebulizer, 1.0 nmol applied per second. This system has been calibrated previously using

both high performance liquid chromatography and an acid-base titration method (Schafer et al., 1985).

Chemicals of reagent grade purity were used in all experiments, with the major suppliers being Aldrich, Pfaltz and Bauer, and Sigma chemical companies.

Recording

Northern grass frogs (Rana pipiens) were anesthetized by a 1 ml injection of 10% urethane and immobilized by injection of 30 mg/kg body weight of d-turbocurarine. The olfactory mucosa was surgically exposed and a Ringer/agar-filled capillary electrode with a tip diameter of 100 um was placed onto the crest of the olfactory mucosa. EOG responses were amplified by a Tektronix AM 502 pre-amplifier. The receptor potential waveforms (EOG's) were displayed on a Tektronix 5113 storage oscilloscope and the waveform amplitude recorded on paper with a pen recorder (Soltec 1242).

Normalization of data and analysis

The average EOG magnitude of the responses to three pulses of the reference odorant, applied just before any inhibitory treatment, was taken as a 100% standard for comparison with subsequent post-treatment responses. Pulses of the reference odorant, IAA, were given at 100 sec intervals both before and after application of inorganic ions or organic agents. For convenience of comparison

between different preparations, the amplitudes of all EOG's (pre- and post-treatment) were expressed in terms of percentage of the pre-treatment baseline level.

Each preparation acted as its own control through the use of pre- and post-tests. In many experiments, both sides of the nose were surgically exposed at the beginning of the experiment. Since each side occupies a separate cavity, two mucosae can be given the same treatment (to provide replicate experiments in the same animal) or given different treatments (to provide both control and experimental conditions in the same animal). It was found that Glad Wrap (a commercial food wrapping) tightly adheres to frog skin and can be used to protect one side of the nose while the other side is being treated. At least three to six replicates were run for each of the agents being tested.

Scanning Electron Microscopy

In an attempt to visualize olfactory receptor molecules, a silicon-labeled amine ($Me_3SiOCH_2NH_2$ or IMMT-9) was applied to frog olfactory mucosae. The rationale was to use an amine which would associate with the olfactory receptor ion channels (Schafer et al., 1985). The presence of the silicon atom would enable localization of the amine using scanning electron microscopy (SEM) and electron dispersive spectroscopy (EDS).

The silicon-labeled amine was applied as a saturated vapor for 15 sec during electrophysiological monitoring. The

olfactory mucosa was then pre-fixed for 5 min *in situ* with 2% glutaraldehyde, then excised and post-fixed for many weeks in 2% glutaraldehyde. The mucosa was dried in alcoholic series and subjected to critical point drying. The sample was examined in an Etec Autoscan SEM equipped with a Tracor-Northern EDS system.

CHAPTER III

RESULTS

Stability of the Preparation and Stimulating System

Typically, after an initial stabilizing period of 30 min following surgery, less than 10 percent variability was seen in electroolfactogram (EOG) magnitudes when the frog olfactory mucosa was stimulated at 100 sec intervals with 0.3 sec pulses of isoamyl acetate (IAA) as a saturated vapor. In virtually all experiments reported here, EOG amplitude in response to IAA varied less than 10 percent during the 20 min period preceding treatment.

Electroofactogram Waveforms

A surface-negative, monophasic voltage change or (EOG) was the typical waveform produced in response to IAA stimulation. Data were not collected from preparations which produced pre-test EOG's with waveforms containing major positive components, since the presence of a major positive component may be correlated with damage or inflammation (Takagi et al., 1969).

Effects of Inorganic Ions and Chelating Agents

The effects of eight inorganic ions and two chelating agents on EOG responses were determined. In each case of the

inorganic ions, 1 nmol of the agent was applied over a 1 sec period. In the case of the chelating agents, 2 nmol of the agent was applied over a 2 sec period. The results of each exposure was monitored for 30-40 min, post-treatment. The results are summarized in Table 1 and the effects of individual agents are shown in Figures 2-9. The order of effectiveness in inhibiting the olfactory response was $Zn^{++}>La^{+++}>Cd^{++}>Ca^{++}>Sr^{++}>Co^{++}>Mg^{++}$. Ba^{++} ion was unique in that it increased, or potentiated EOG responses.

The chelating agents, ethyleneglycol tetraacetic acid (EGTA) and ethylenediamine tetraacetic acid (EDTA) inhibited EOG responses by 45 percent and 18 percent (over a 20 min period), respectively. Ethyleneglycol tetraacetic acid, a specific calcium chelator, inhibited more strongly than EDTA (Figures 10-11).

Local Anesthetics

The local anesthetics dibucaine, tetracaine, and procaine were each applied as aerosols for a 2 sec period, exposing the olfactory mucosa to 1.0 nmol of the anesthetic. The order of effectivness in inhibiting EOG responses was dibucaine>tetracaine>procaine. The results of these experiments are summarized in Table 1, with the individual experiments shown in Figures 12-14.

Silicon-Labeled Amine

Preliminary experiments on channel localization were

performed using a silicon-labeled amine, 2-(Trimethylsiloxy)ethylamine ($(CH_3)_3SiOH_2CH_2CH(CH_3)_2$), which is believed to associate with ion channels (Schafer, personal communication). The amine, when applied in the vapor phase over a 15 sec time period, proved to be very inhibitory, reducing EOG responses to less than 10 percent of the pretreatment magnitude (Table 1, Figure 15). Preliminary attempts to localize the silicon label in tissue using scanning electron microscopy and electron dispersive spectroscopy were inconclusive, although silicon was detected in the tissue. However, the exact location of the label has not yet been determined.

Controls

Distilled water, which was the solvent used to dissolve all agents, was found to be non-inhibitory. However, a better control would be water containing a divalent cation. Since Mg^{++} was found to be non-inhibitory to olfactory responses, it is the most appropriate control for these experiments (Figure 8).

TABLE I

INHIBITORY ACTIVITY OF INORGANIC IONS AND ORGANIC AGENTS

Agent	N	Percent Inhibition at 10 min	Percent Inhibition at 20 min
$ZnCl_2$	4	89 +/- 4	84 +/- 4
$LaCl_3$	3	73 +/- 2	54 +/- 3
$CdCl_2$	4	54 +/- 7	39 +/- 6
$CaCl_2$	5	33 +/- 5	20 +/- 14
$CoCl_2$	4	18 +/- 13	1 +/- 5
$SrCl_2$	5	15 +/- 7	1 +/- 4
$MgCl_2$	6	8 +/- 12	0 +/- 2
$BaCl_2$	4	-4 +/- 16	-18 +/- 30
EGTA	3	47 +/- 7	34 +/- 3
EDTA	4	19 +/- 13	10 +/- 8
Dibucaine	4	59 +/- 4	44 +/- 4
Tetracaine	5	36 +/- 7	15 +/- 8
Procaine	5	12 +/- 6	7 +/- 2
IMMT-9	3	81 +/- 5	63 +/- 13

Figure 1. Stimulating and recording system.

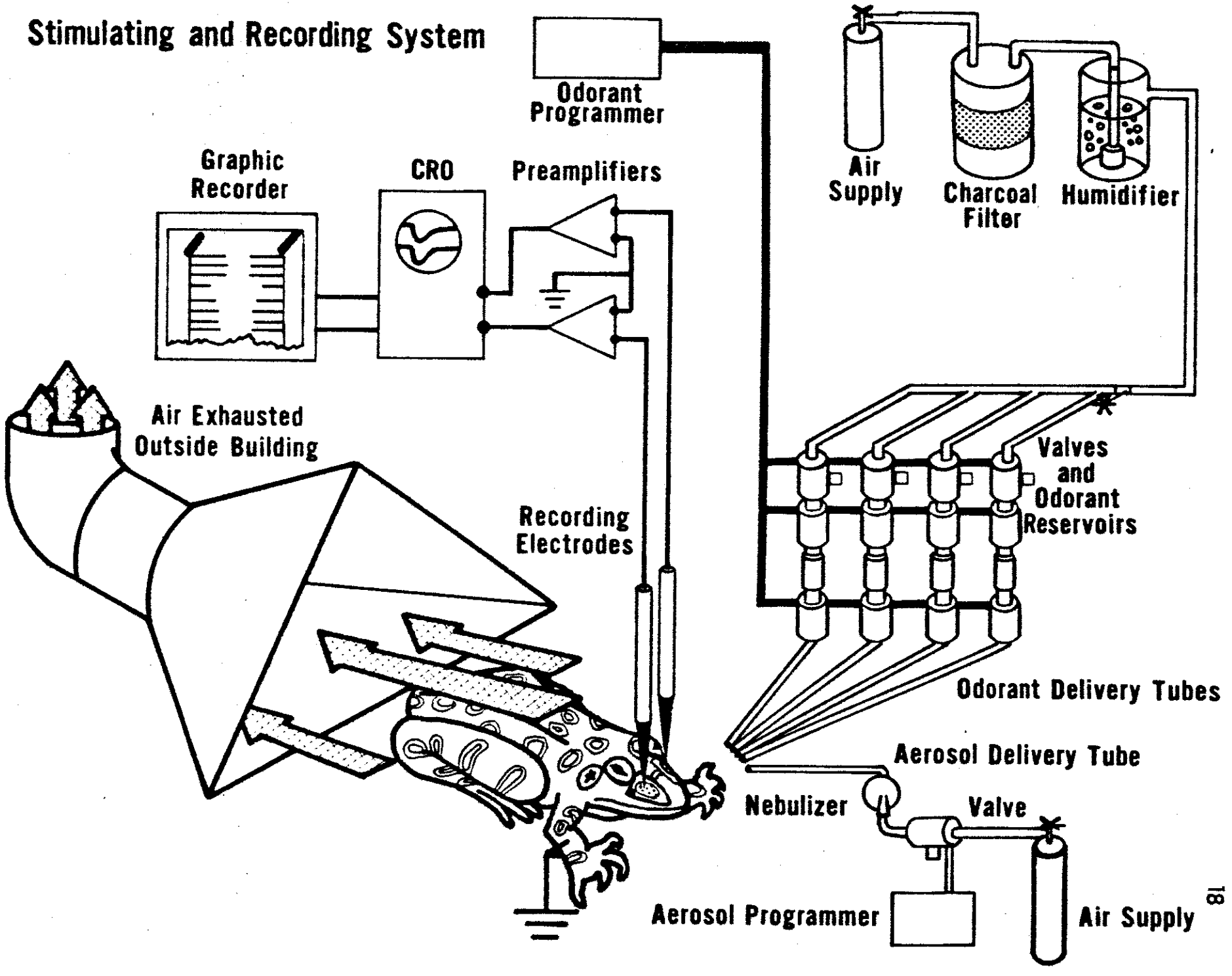

Stimulating and Recording System
Odorant Programmer
Air Supply
Charcoal Filter
Humidifier
Graphic Recorder
CRO
Preamplifiers
Air Exhausted Outside Building
Recording Electrodes
Valves and Odorant Reservoirs
Odorant Delivery Tubes
Aerosol Delivery Tube
Nebulizer
Valve
Aerosol Programmer
Air Supply

Figure 2. Effect on EOG responses of an exposure to 1 nmol of $ZnCl_2$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 8.0% of the pretreatment level, followed by a very slow rate of recovery.

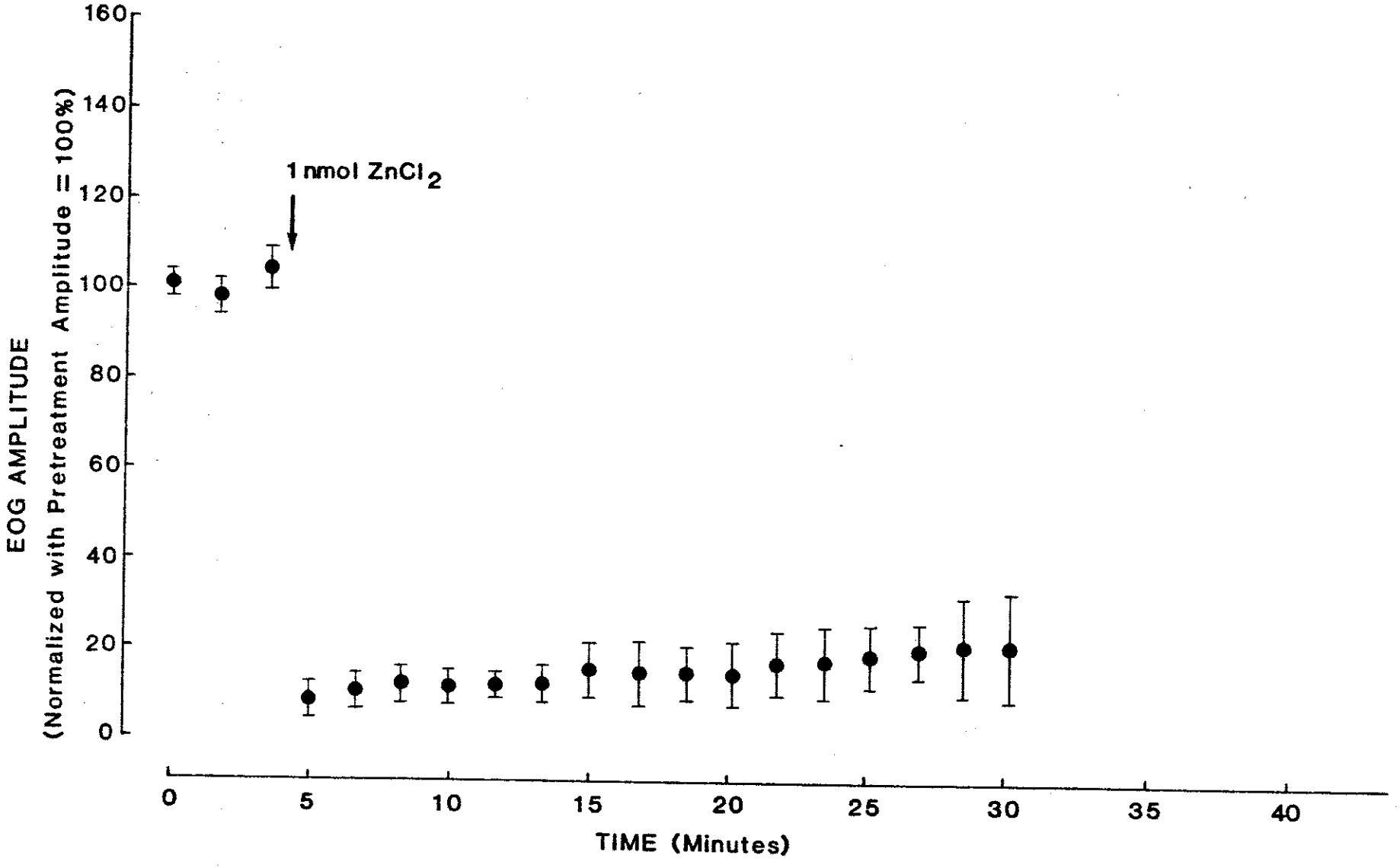

EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
160
140
120
100
80
60
40
20
0
1 nmol $ZnCl_2$
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 3. Effect on EOG responses of an exposure to 1 nmol of $LaCl_3$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 16.0% of the pretreatment level, followed by a very slow rate of recovery.

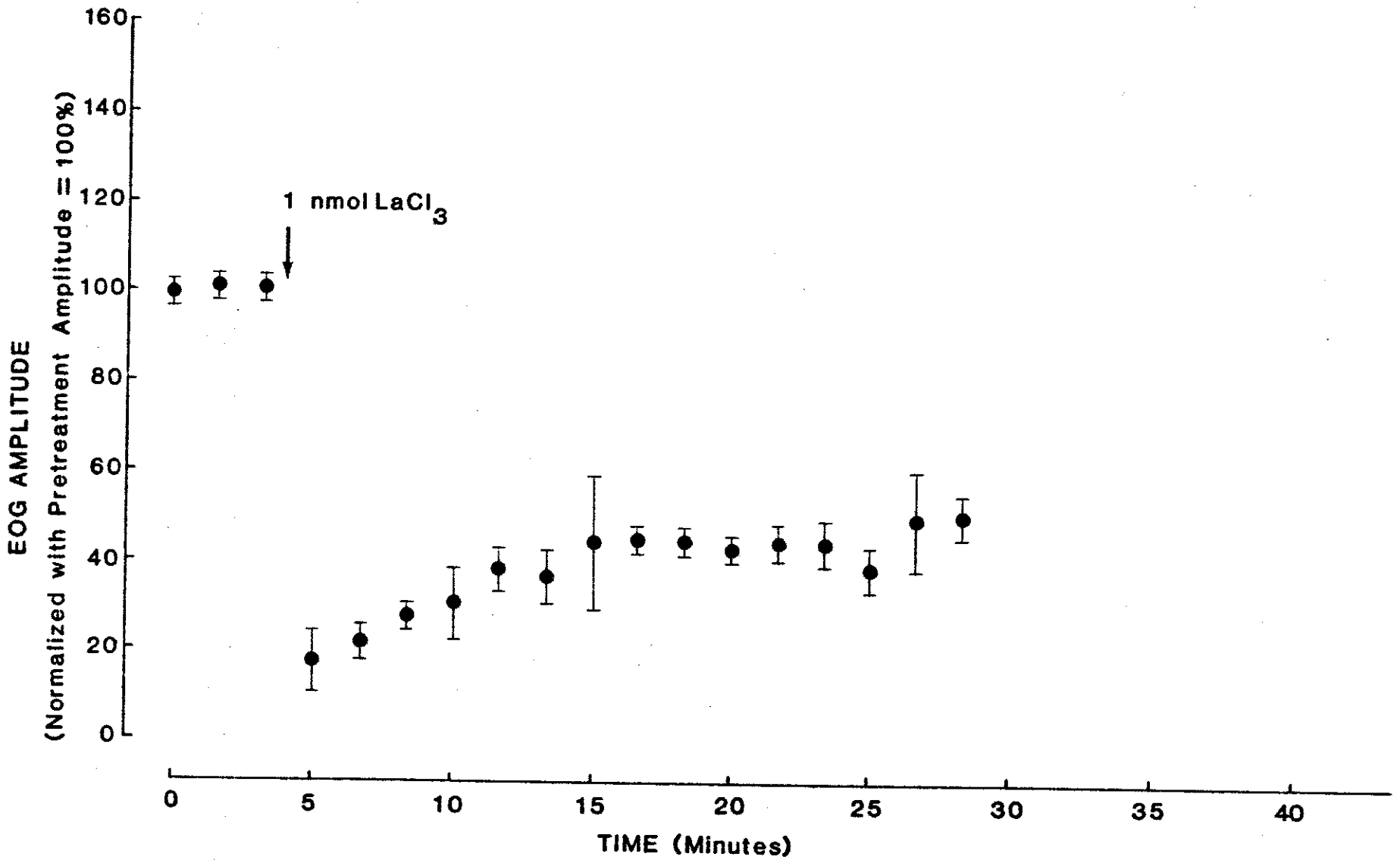

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
1 nmol LaCl3
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 4. Effect on EOG responses of an exposure to 1 nmol of $CdCl_2$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 18.0% of the pretreatment level, followed by a moderate rate of recovery.

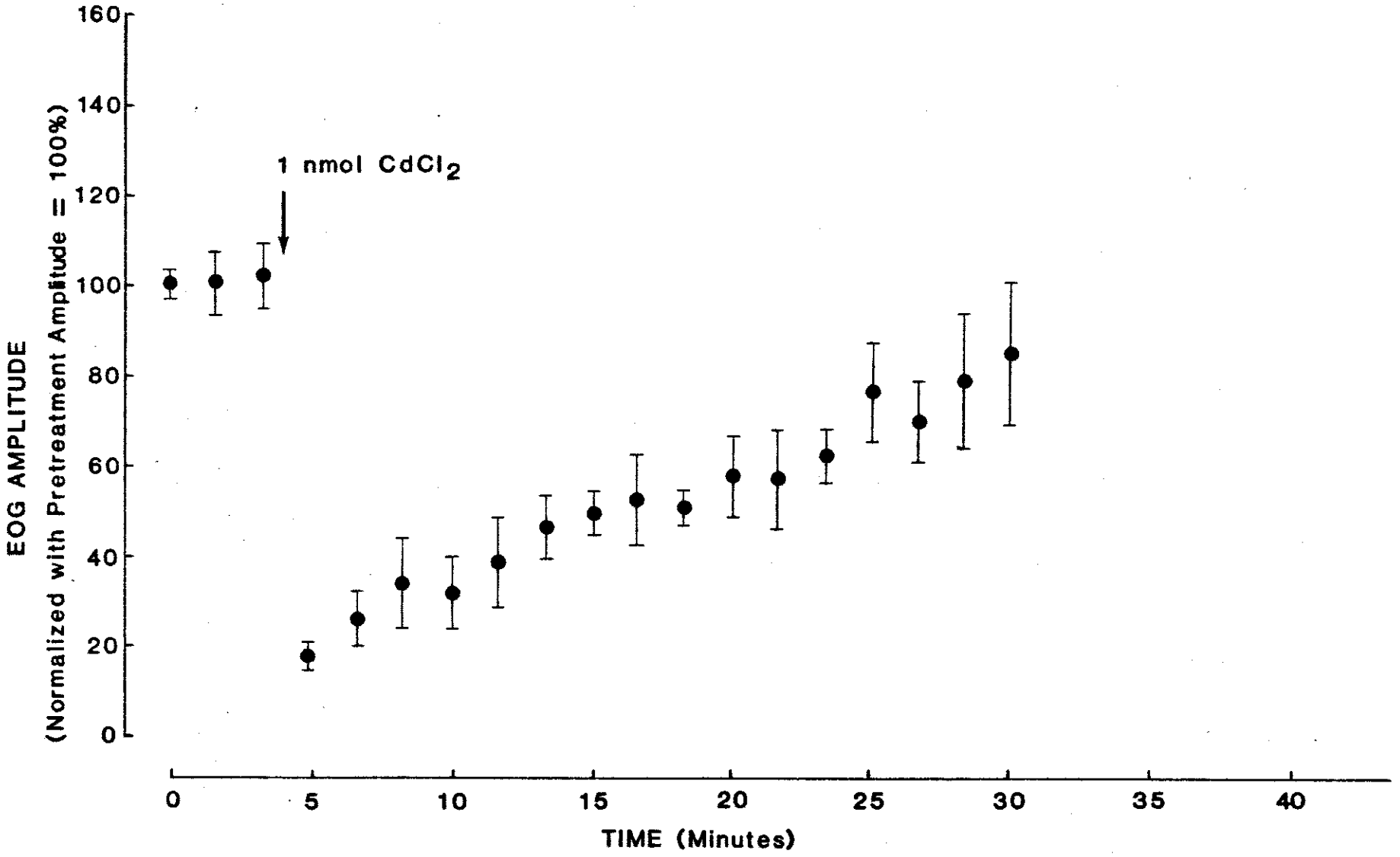

EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
160
140
120
100
80
60
40
20
0
1 nmol $CdCl_2$
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 5. Effect on EOG responses of an exposure to 1 nmol of $CaCl_2$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 60.0% of the pretreatment level, followed by a moderate rate of recovery.

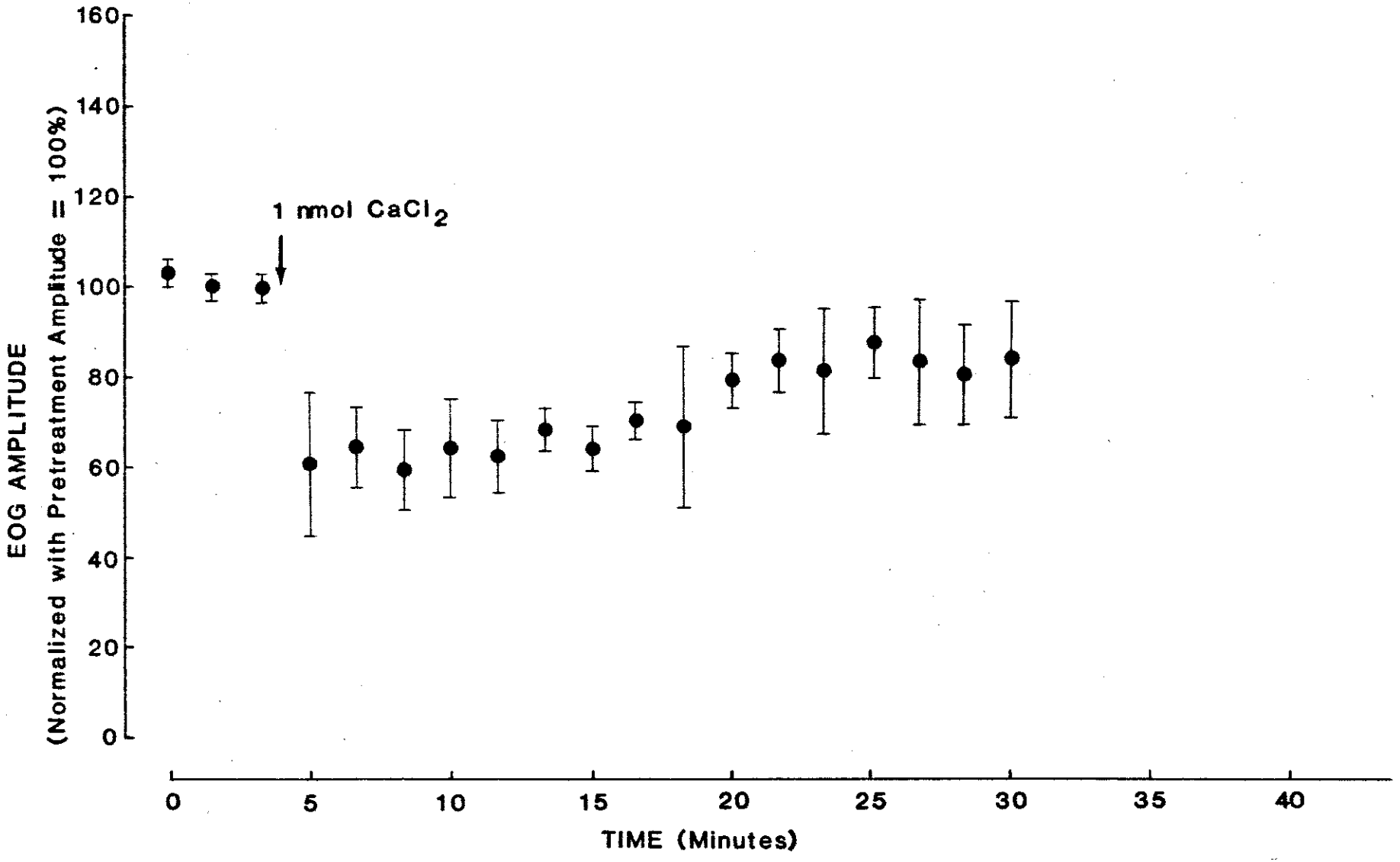

EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
160
140
120
100
80
60
40
20
0
1 mmol $CaCl_2$
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 6. Effect on EOG responses of an exposure to 1 nmol of $CoCl_2$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 77.0% of the pretreatment level, followed by a rapid rate of recovery.

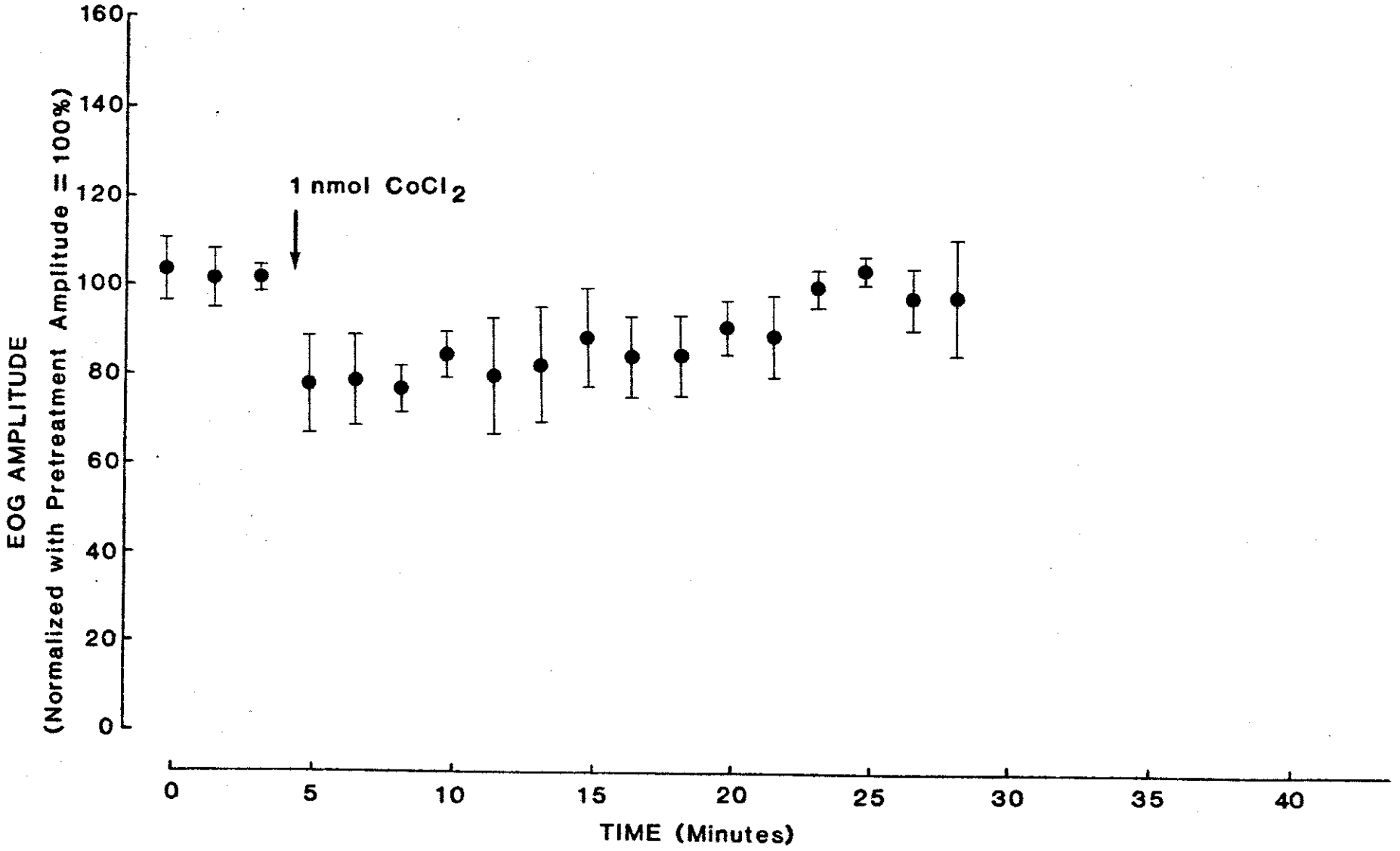

EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
160
140
120
100
80
60
40
20
0
1 nmol $CoCl_2$
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 7. Effect on EOG responses of an exposure to 1 nmol of $SrCl_2$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 81.0% of the pretreatment level, followed by a rapid rate of recovery.

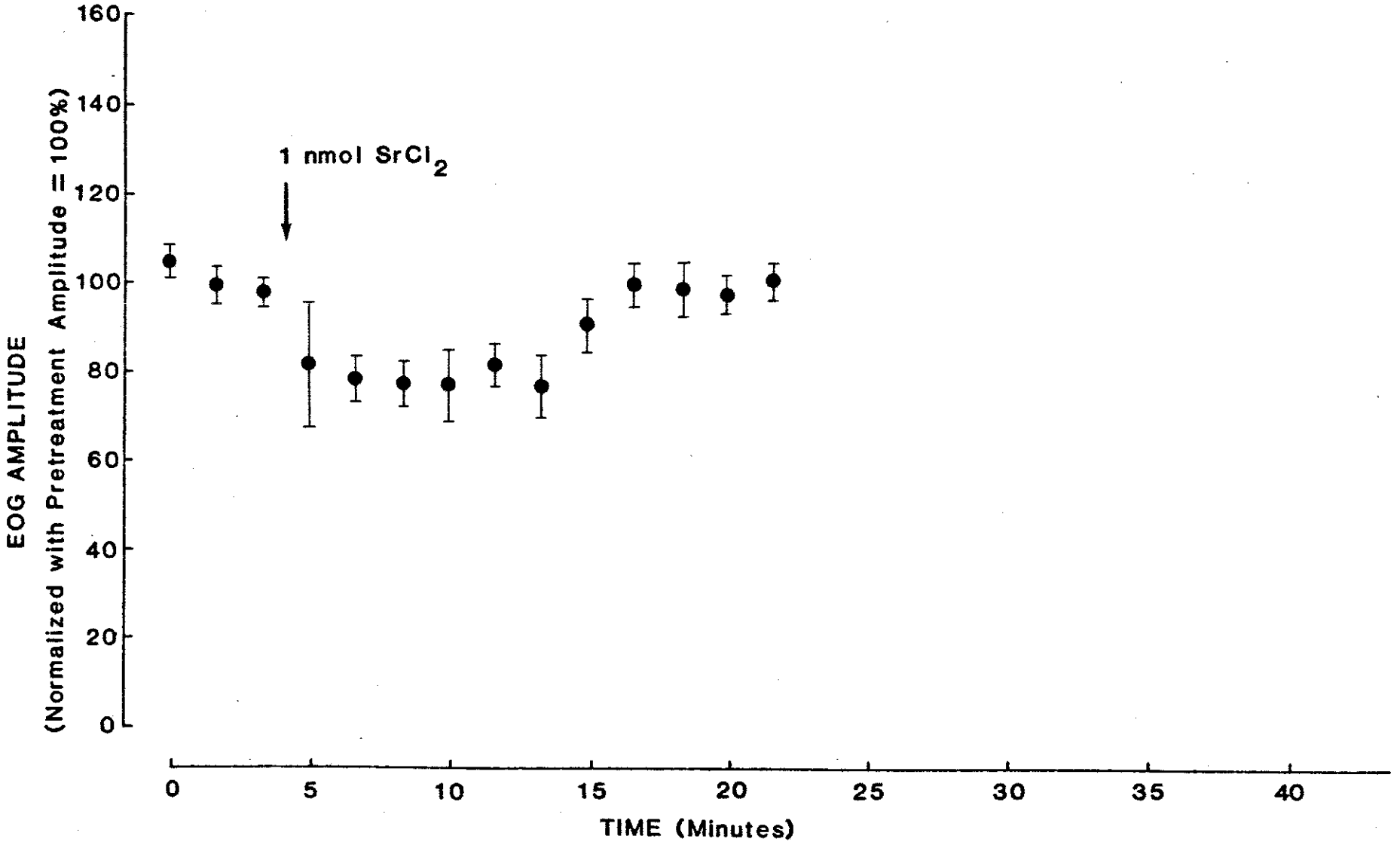

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
1 nmol $SrCl_2$
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 8. Effect on EOG responses of an exposure to 1 nmol of $MgCl_2$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were not reduced by any appreciable degree.

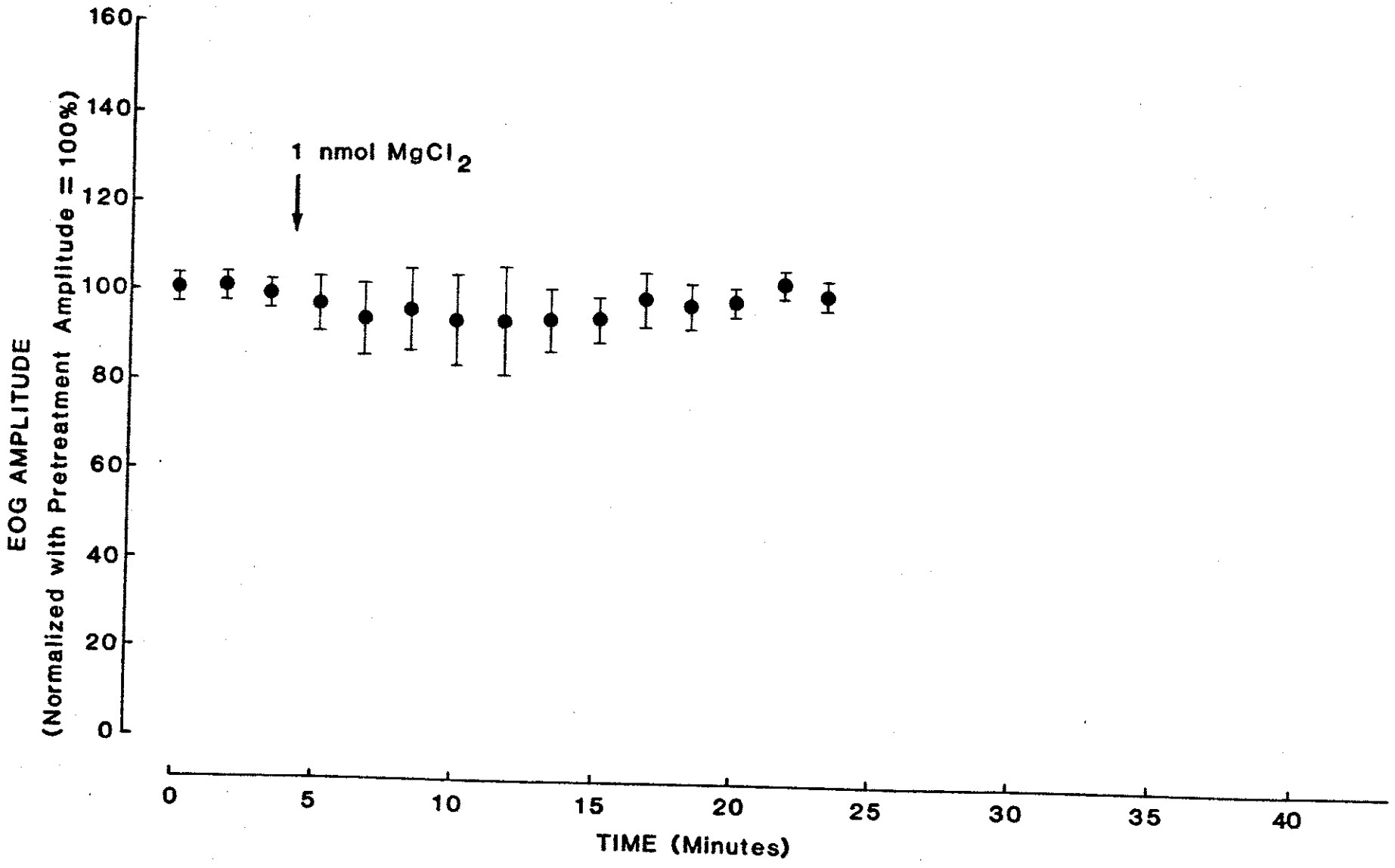

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
1 nmol MgCl2
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 9. Effect on EOG responses of an exposure to 1 nmol of $BaCl_2$ given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially increased to 104% of the pretreatment level, followed by a rise to 120%.

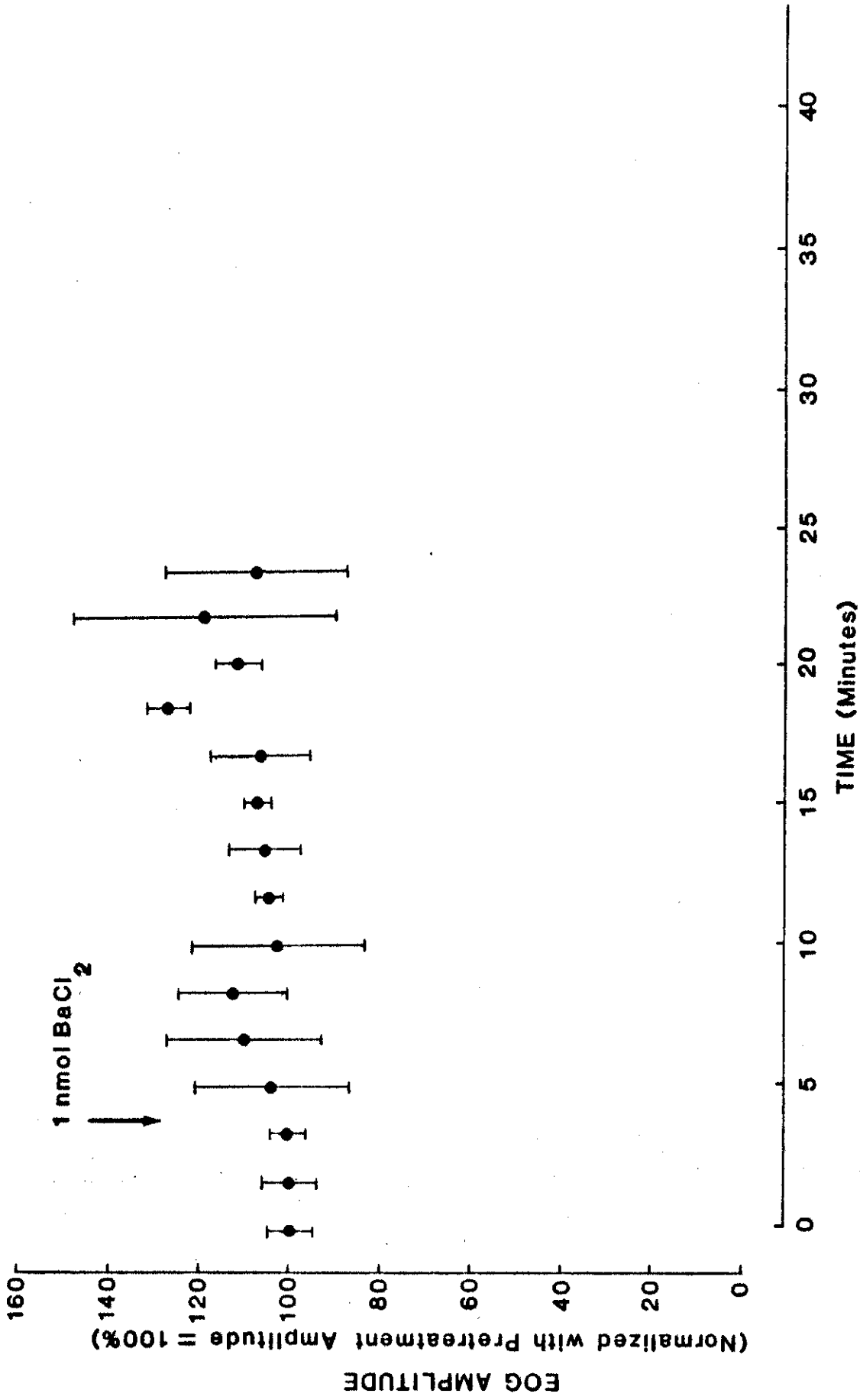

EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
160
140
120
100
80
60
40
20
0
1 nmol $BaCl_2$
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 10. Effect on EOG responses of an exposure to 2 nmol of EGTA given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 48.0% of the pretreatment level, followed by a moderate rate of recovery.

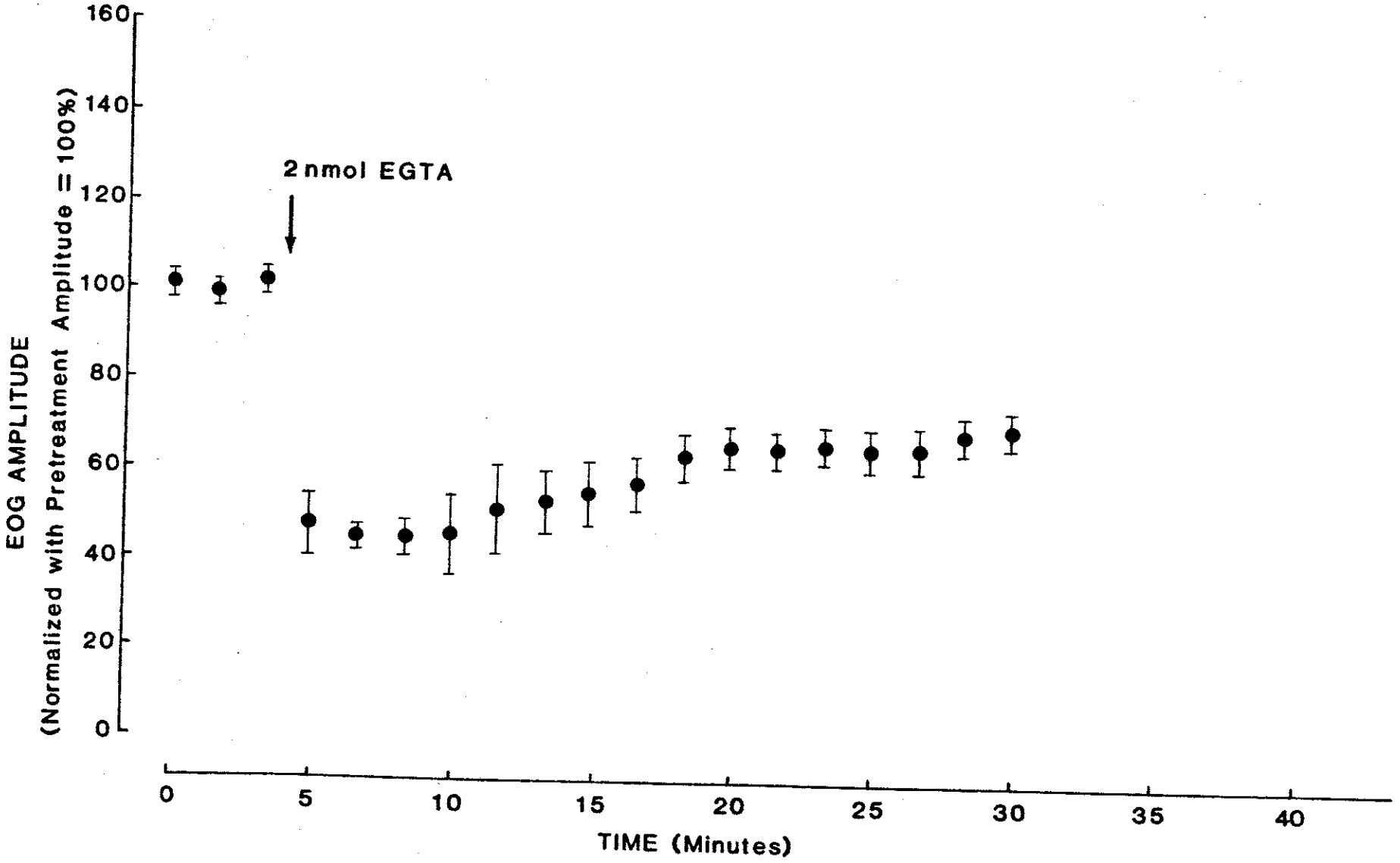

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
2 nmol EGTA
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 11. Effect on EOG responses of an exposure to 2 nmol of EDTA given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 65.0% of the pretreatment level, followed by a rapid rate of recovery.

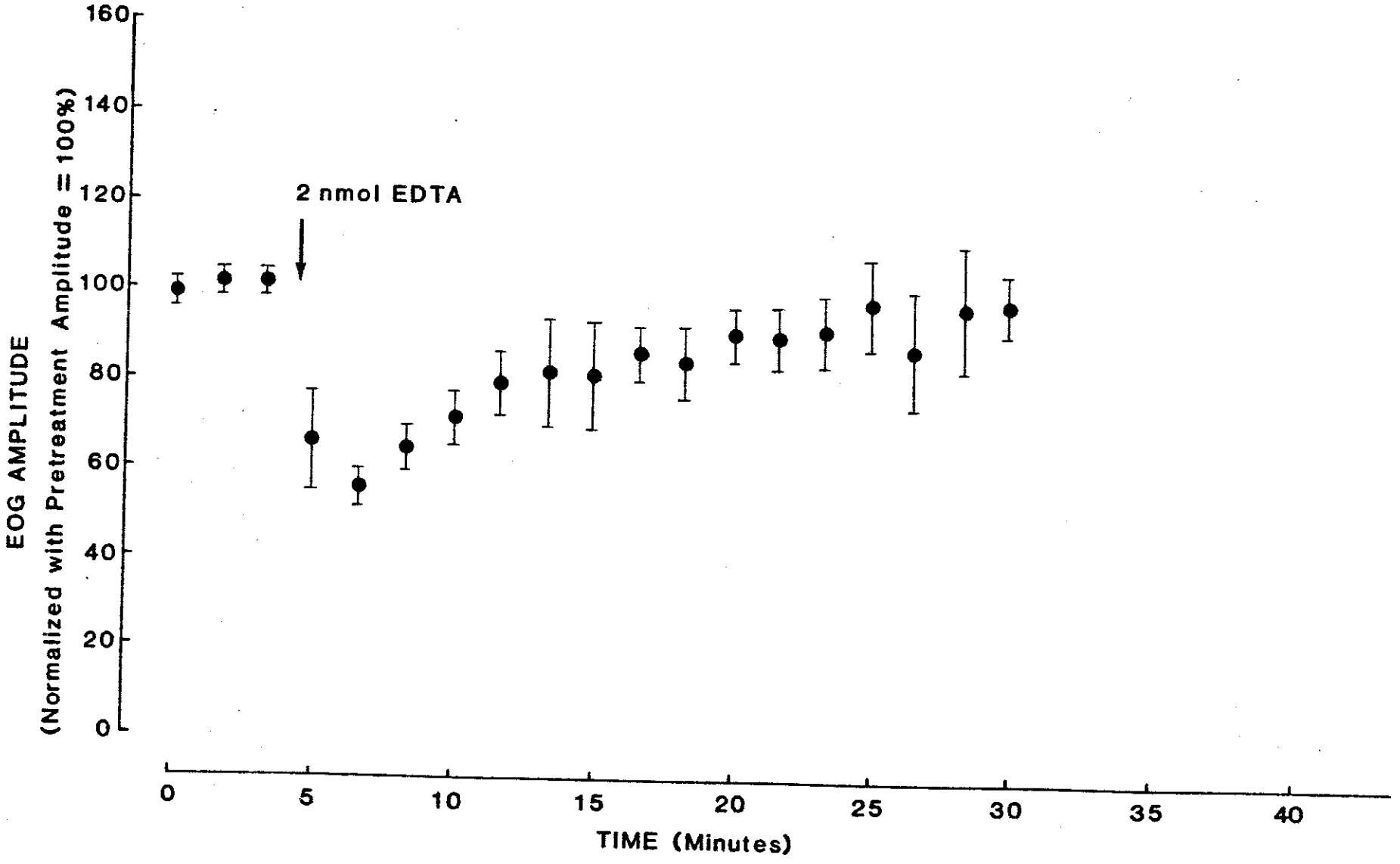

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
2 nmol EDTA
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 12. Effect on EOG responses of an exposure to 1 nmol of dibucaine given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 19.0% of the pretreatment level, followed by a slow rate of recovery.

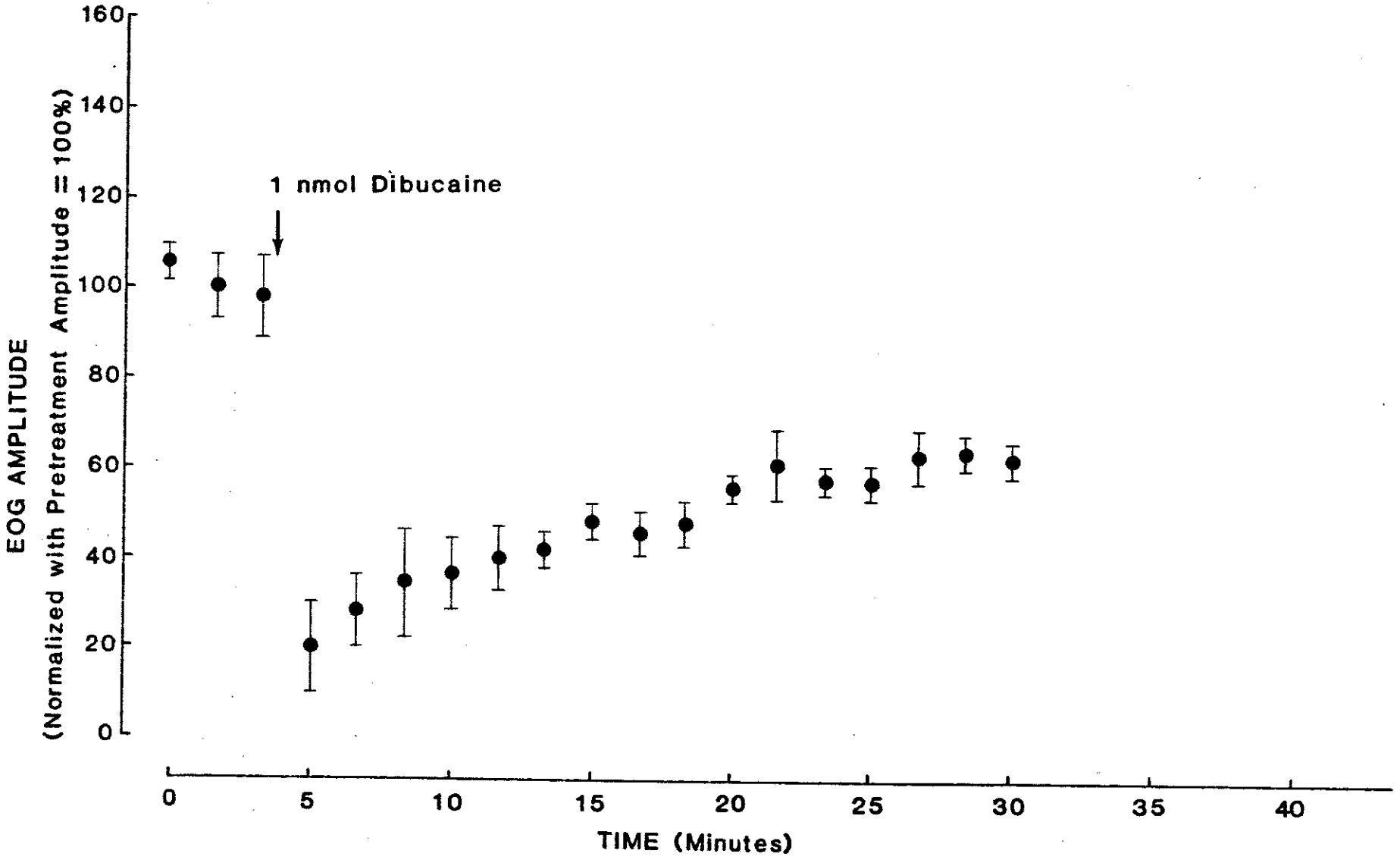

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
1 nmol Dibucaine
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 13. Effect on EOG responses of an exposure to 1 nmol of tetracaine given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 41.0% of the pretreatment level, followed by a moderate rate of recovery.

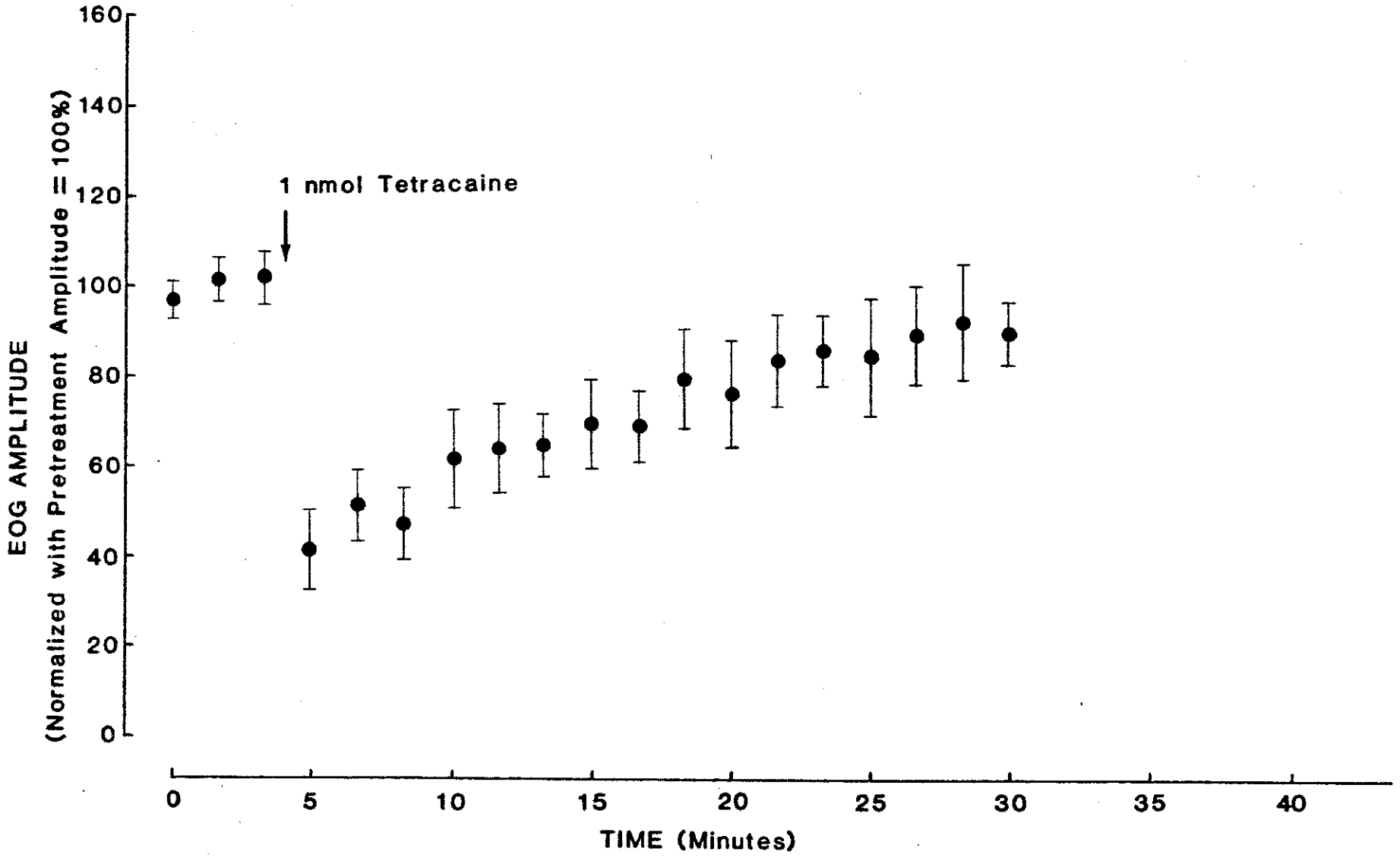

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
1 nmol Tetracaine
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 14. Effect on EOG responses of an exposure to 1 nmol of procaine given over a 2 sec period. Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 91.0% of the pretreatment level, followed by a rapid rate of recovery.

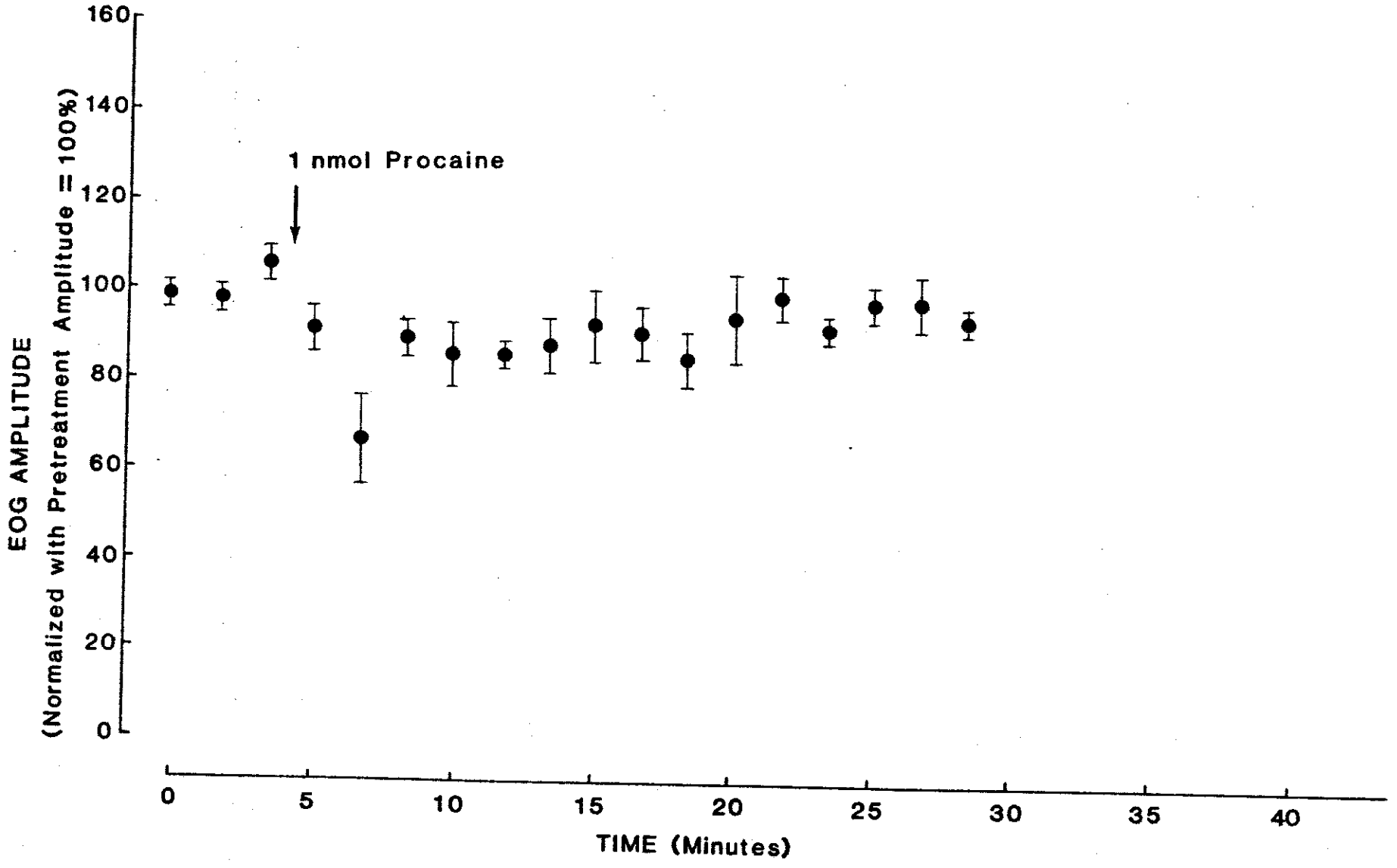
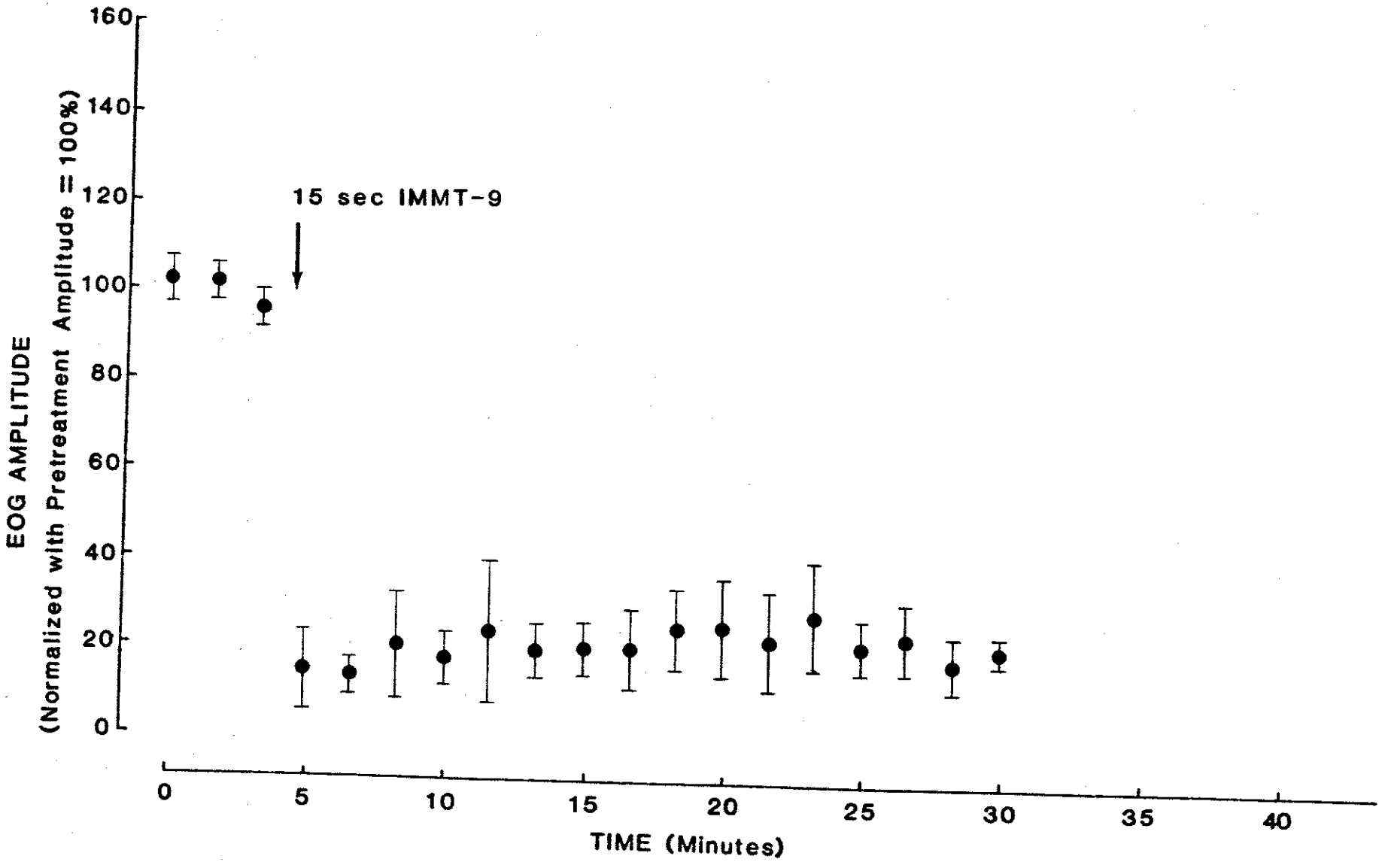

160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
1 nmol Procaine
0
5
10
15
20
25
30
35
40
TIME (Minutes)

Figure 15. Effect on EOG responses of an exposure to 15 sec (vapor) of IMMT-9($CH_3COOCH_2CH_2CH(CH_3)_2$). Error bars indicate +/- 1 standard deviation. Electroolfactogram responses were initially reduced to 14.0% of the pretreatment level, followed by a very slow rate of recovery.


160
140
120
100
80
60
40
20
0
EOG AMPLITUDE
(Normalized with Pretreatment Amplitude = 100%)
15 sec IMMT-9
0
5
10
15
20
25
30
35
40
TIME (Minutes)

CHAPTER IV

DISCUSSION

Olfactory responses in the frog, Rana pipiens, are inhibited by inorganic cations and local anesthetics which are known to interfere with calcium channel-mediated processes in other tissues. The order of effectiveness of di- and trivalent ions in inhibiting frog olfactory responses ($Zn^{++}>La^{+++}>Cd^{++}>Ca^{++}>Sr^{++}>Co^{++}>Mg^{++}$) is similar to the results of Hagiwara and Takahashi (1967) in work on barnacle muscle fibers, where the order of effectiveness in inhibiting calcium channel-dependent action potentials is $La^{+++}>Zn^{++}>Co^{++}>Ca^{++}>Mg^{++}>Sr^{++}$. These all or none action potentials are pure "calcium spikes," in that they depend solely on Ca^{++} permeability changes as opposed to most other muscle membranes where Na^{+} permeability changes are predominant in generating action potentials (Hagiwara and Takahashi (1967). These researchers suggested that the divalent and trivalent ions suppress calcium-dependent action potentials by competing with Ca^{++} for "sites on the membrane" (Hagiwara and Takahashi, 1967).

The results of inorganic ion treatment of the frog olfactory mucosa also parallel the results of Suzuki (1978)

who examined the receptor potentials of single olfactory cells in the lamprey, Entosphenus japonicus. He varied the extracellular concentrations of Na^+ and K^+, and found no change in receptor responses. These results are also consistent with those of Schafer et al. (1985) who showed that application of tetrodotoxin and tetraethylammonium ion, which are respectively Na^+ and K^+ channel blockers, had no effect on the olfactory responses of the frog.

Suzuki also found that EGTA, a specific calcium chelator, reversibly suppressed receptor potentials in the lamprey. In my experiments with the frog, the specific calcium chelator, EGTA, inhibited olfactory responses more strongly than the non-specific chelator, EDTA. The effects of both chelators were reversible in the frog, just as in Suzuki's experiments with the lamprey. Suzuki also examined the effects of La^{+++} and Co^{++} on lamprey olfactory receptor responses, finding that Co^{++} inhibited by 50 percent, while La^{+++} totally abolished all receptor potentials. Likewise, in the frog, olfactory receptor potentials were inhibited by La^{+++} far more than by Co^{++}. La^{+++}, while not totally abolishing olfactory responses, inhibited EOG responses by 70% (measured five minutes after treatment). By contrast, Co^{++} inhibited by only 20 percent (again measured five minutes after treatment).

Although Ca^{++} itself was moderately inhibitory to frog

olfactory responses, this is not a paradox. Reuter (1983) showed that excess Ca^{++} can interefere with the function of calcium-selective channels. The assumption is that a larger initial calcium current, produced by excess Ca^{++}, produces more rapid inactivation of calcium channels.

It is important to note that frog olfactory responses were potentiated by the application of Ba^{++}, increasing to 140% of the pretreatment level by 15 minutes after treatment. Ba^{++} potentiates calcium channel-mediated processes in other tissues, such as rat pituitary tissue, where Ba^{++} produces an even larger current than Ca^{++} itself as measured by cell clamp methods Hagiwara and Ohmori (1982). The effect of Ba^{++} is unique among the inorganic ions tested because it potentiated frog EOG responses rather than inhibited them.

The effects of the local anesthetics, dibucaine, tetracaine, and procaine were examined, since their mechanism of action may be to disrupt or displace Ca^{++} by dissolving in the lipid of the membrane and increasing the distance separating components of the Ca^{++} binding sites (Low et al., 1978). It was found that the order of effectiveness in inhibiting frog olfactory responses is dibucaine>tetracaine>procaine. Similarly, several investigators have shown that the order of effectiveness in displacing Ca^{++} from membrane binding sites in erythrocytes and model phospholipid bilayers is

dibucaine>tetracaine>procaine, the same order in which they inhibit EOG responses in the frog (Papahadjopoulos, 1971; Low et al., 1978).

Attempts were made to label the olfactory receptor/ionophore complex with the silicon-labeled amine, IMMT-9. Schafer et al. (1985) showed that many amines are highly inhibitory to olfactory responses in the frog, and suggested that this may be due to the ability of the protonated form of the amine to interfere with the ion channels associated with the olfactory receptors. This was the basis for using the silicon- labeled amine as a marker for the receptor/ionophore complex. Application of a 15 sec pulse of the amine proved to be highly inhibitory to olfactory responses. Attempts to localize the amine label using SEM and EDS were inconclusive, although silicon was detected in the tissue.

Summary

1. Olfactory responses in the frog are inhibited by di- and trivalent ions which interfere with calcium channel-mediated processes.

2. Barium ion potentiates olfactory responses in the frog and is known to carry current in calcium channels better than Ca^{++} itself.

3. Chelators of divalent ions (EGTA and EDTA) inhibit olfactory responses in the frog.

4. Local anesthetics which may interfere with calcium channel function also inhibit frog olfactory responses.

5. It is likely that Ca^{++} plays a role in olfactory transduction, either as a current carrier or through participation in a second messenger process, or both.

BIBLIOGRAPHY

Articles

Browning, J. and D. Nelson 1976 Amphipathic amines affect membrane excitability in Paramecium: Role for bilayer couple. Proc. Natl. Acad. Sci., U.S.A., 73: 452-456.

Chen, Z. and D. Lancet 1984 Membrane proteins unique to verterbrate olfactory cilia. Proc. Natl. Acad. Sci., U.S.A., 81: 1859-1863.

Dunlap, K. and R. Eckert 1984 Calcium channels in the ciliary membrane of Paramecium. J. Cell Bio., 70: 245a.

Dute, R. and C. Chung 1976 Ultrastructure of the proximal region of somatic cilia in Paramecium tetraurelia. J. Cell Bio., 78: 451-464.

Fesenko, E., V. Novoselov, L. Krapivinskaya, N. Mjasoedov and J. Zolotarev 1983 Some biochemical charateristics of a possible receptor for amino acids from the olfactory epithelium of the skate Dasyatis pastinaca and carp Cyprinus carpio. Biochimia et Biophysica Acta, 59: 250-256.

Getchell, T., G. Heck, J. DeSimone and S.Price 1981 The location of olfactory receptor sites. J. Biophys., 29: 397-412.

Gilula, N. and P. Satir 1972 The ciliary necklace. J. Cell Bio., 53: 494-508.

Glossmann, H., D. Ferry, F. Lubbecke, R. Mewes and F. Hofmann 1982 Calcium channels: Direct identification with radioligand binding studies. Trends in Pharm. Sciences, 11: 431-437.

Hagiwara, S. and K. Takahshi 1967 Surface density of calcium ions and calcium spikes in the barnacle muscle fiber membrane. J. Gen. Physiol., 50: 583-601.

Hagiwara, S. and O. Harunori 1982 Studies of calcium channels in rat clonal pituitary cells with patch electrode voltage clamp. J. Physiol., 331: 231-252.

Kleene, S. and R. Gesteland 1981 Dissociation of frog olfactory epithelium with N-ethylmaleimide. Brain Research, 229: 536-540.

Low, P., D. Lloyd, T. Stein and J. Rogers 1978 Calcium displacement by local anesthetics. J. Biological Chem., 254: 4119-4125.

Mackay-Sim, A. and J. Kubie 1981 The salamander nose: a model system for the study of spatial coding of olfactory ouality. Chem. Senses, 6: 249-257.

Mair, R., R. Gesteland and D. Blank 1982 Changes in morphology and physiology of olfactory receptor cilia during development. Neuroscience, 7: 3091-3104.

Mason, J., L. Clark and T. Morton 1984 Selective deficits in the sense of smell caused by chemical modification of the olfactory epithelium. Science, 226: 1092-1094.

Menco, M. 1980 Qualitative and quantitative freeze-fracture studies on olfactory and nasal respiratory structures of frog, ox, rat, and dog. Cell Tissue Research, 207: 183-209.

Menco, M., G. Dodd, M. Davey and L. Bannister 1976 Presence of membrane particles in freeze-etched bovine olfactory cilia. Nature, 263: 597-599.

Menevse, A., G. Dodd and T. Poynder 1977 Evidence for the specific involvement of cAMP in the olfactory transduction

process. Biochem. and Biophys. Res. Comm., 77: 671-677.

Miragall, F. 1983 Evidence for orthogonal arrays of particles in the plasma membrane of olfactory and vomeronasal sensory neurons of vetebrates. J. Neurocytology, 12: 567-576.

Moran, D., F. Varela and J. Rowley 1977 Evidence for active role of cilia in sensory transduction. Proc. Natl. Acad. Sci., U.S.A. 74: 793-797.

Moulton, D. and L. Beidler 1967 Structure and function in the peripheral olfactory system. Phys. Reviews, 47: 1-52.

Naitoh, Y. and H. Kaneko 1972 Control of ciliary activities by ATP and divalent cations in triton-extracted models of Paramecium caudatum. J. Exp. Biol., 58: 657-676.

Papahadjopoulos, D. 1971 Studies on the mechanism of action of local anesthetics with phospholipid model membranes. Biochim. Biophys. Acta, 265: 169-186.

Reese, T. 1965 Olfactory cilia in the frog. J. Cell Bio., 25: 209-230.

Reuter, H. 1983 Calcium channel modulation by

neurotransmitters, enzymes, and drugs. Nature, 301: 569-574.

Schafer, R., S. Fracek, D. Criswell and K. Brower 1984a Protection of olfactory responses from inhibition by ethyl bromoacetate, diethylamine, and other chemically active odorants by certain esters and other compounds. Submitted to Chemical Senses.

Schafer, R., D. Criswell, S. Fracek and K. Brower 1984b Olfactory studies using ethyl bromoacetate and other chemically active odorants. Submitted to Chemical Senses.

Schafer, R., S. Fracek, B. Winegar and K. Brower 1985 Olfactory transduction is inhibited by amine vapors and a lipophilic ion channel ligand. Submitted to Chemical Senses.

Suzuki, H. 1978 Effects of different ionic enviroments on the responses of the single olfactory receptors in the lamprey. Comp. Biochem. Physiol., 61: 461-467.

Synder, S. 1984 Drug and neurotransmitter receptors in the brain. Science, 224: 22-31.

Usukura, J. and E. Yamada 1978 Observations on the cytolemma of the olfactory receptor cell of the newt. Cell Tiss. Res., 31: 83-98.

Volpi, C., R. Shaafi, P. Epstein, D. Andrenyak, and M. Feinstein 1981 Local anesthetics, mepacrine, and propranolol are antagonists of calmodulin. Natl. Acad. Sci. U.S.A., 2: 795-799.

Yammamoto, T., A. Tonosaki and T. Kurosawa 1965 Electron microscope studies on the olfactory epithelium in frogs. Acta Anat. Nippon, 40: 342-353.

Books

Price, S. 1981 Receptor proteins in vertebrate olfaction: Biochemistry of taste and olfaction. Academic Press, Inc., New York. pp. 69-84.

Rhein, L. and R. Cagan 1981 Role of cilia in olfactory recognition: Biochemistry of taste and olfaction. Academic Press, Inc., New York. pp. 47-67.

Takagi, S., K. Aoki, M. Iino and T. Yajima 1969 The electropositive potential in the normal and degenerating olfactory epithelium: Olfaction and taste. Rockefeller Univ. Press, New York. pp. 92-108.