Journal of Physiology (1992), **453**, *pp.* 133–166
With 12 figures
Printed in Great Britain

EFFECTS OF MUSCARINE ON SINGLE RAT ADRENAL CHROMAFFIN CELLS

BY ALAN NEELY* AND CHRISTOPHER J. LINGLE†

From the Department of Anesthesiology, Washington University School of Medicine, 660 South Euclid Avenue, St Louis MO 63110, USA

(*Received 26 September 1991*)

SUMMARY

1. The action of muscarine on membrane currents and cytosolic calcium (Ca^{2+}) of dissociated rat adrenal chromaffin cells was investigated using standard whole-cell voltage-clamp techniques and microfluorimetry of unclamped single cells.

2. In cells held at a constant holding potential negative to −40 mV, brief (5–10 s) applications of muscarine produced a transient activation of outward current. The activation of this current by muscarine also occurs in the presence of 5 mM-Co^{2+}.

3. The outward current activated by muscarine at holding potentials negative to about −40 mV is blocked over 90% by either 200 μM-curare or 200 nM-apamin. One millimolar TEA produces variable blocking effects at such potentials.

4. The outward current activated by muscarine is transient even in the continuing presence of muscarine. Complete recovery between pairs of muscarine applications occurs over a 1–2 min period. If sufficient time was allowed for recovery between muscarine applications, the muscarine-activated outward current could be reliably elicited in dialysed cells for periods of 20–30 min.

5. Voltage ramps were used to examine effects of muscarine on currents over a range of membrane potentials. Over all potentials, muscarine activates a relatively voltage-independent component which is blocked almost completely by 200 nM-apamin and by 200 μM-curare. At potentials negative to about −40 mV, the apamin- and curare-sensitive current accounts for virtually all muscarine-activated current. This current appears to correspond to a Ca^{2+}-activated, voltage-independent current found in these cells. Effects of muscarine on currents activated at potentials positive to 0 mV are complex. At potentials above 0 mV, muscarine can produce either an activation or an inhibition of outward current. The outward current activated at positive potentials was primarily voltage dependent and blocked by 1 mM-TEA. However, in some cells activation of voltage-dependent current was not observed and, in such cases, muscarine produced an inactivation of the voltage-dependent component of current. The inactivation of outward current could also be observed in the presence of 5 mM-Co^{2+} indicating that the inactivation does not occur secondarily to an effect of muscarine on Ca^{2+} current. The possibility is discussed that the

* Present address: Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA.

† To whom correspondence should be addressed.

MS 8758

inactivation of outward current occurs as a result of intrinsic inactivation properties of the voltage-dependent Ca^{2+}-dependent K^+ current. According to this hypothesis, the extent to which inactivation of voltage-dependent outward current is observed depends on the magnitude of the muscarine-induced cytosolic Ca^{2+} elevation and the level of depolarization of the cell.

6. Muscarine application to single Fura-2-loaded chromaffin cells produced a transient increase in cytosolic Ca^{2+}. The muscarine-induced increase in Ca_i^{2+} also occurred following removal of Ca^{2+} from the extracellular saline or following addition of 2 mM-cobalt.

7. The results are consistent with the view that a muscarine-triggered release of Ca^{2+} from intracellular stores can activate two components of Ca^{2+}-dependent K^+ current. Furthermore, some mechanism occurs by which the voltage-dependent component of Ca^{2+}-dependent current can undergo inactivation during the muscarine-induced elevation of Ca^{2+}.

INTRODUCTION

The primary trigger for secretion of catecholamines from chromaffin cells of the adrenal gland is acetylcholine released from splanchnic nerves (Feldberg, Minz & Tsudzimura, 1934; Douglas & Rubin, 1961; Douglas, 1975; Wakade, 1981). In many species both nicotinic (nAChR) and muscarinic (mAChR) acetylcholine receptors on chromaffin cells contribute to activation of secretion of catecholamines (Kirpekar, Prat & Shiavone, 1982; Role & Perlman, 1983; Wakade & Wakade, 1983; Warashina, Fujiwara & Shimoji, 1989). Recent evidence now indicates that nAChRs and mAChRs trigger secretion by fundamentally distinct mechanisms. In particular, nAChR-mediated secretion appears to result directly from depolarization of the chromaffin cell with a consequent transient increase in action potential frequency and/or opening of voltage-dependent calcium (Ca^{2+}) channels (Douglas, Kanno & Sampson, 1967; Kidokoro, Miyazaki & Ozawa, 1982; Fenwick, Marty & Neher, 1982*a*, *b*; Hirano, Kidokoro & Ohmori, 1987). In contrast, mAChR activation triggers an elevation of cytosolic Ca^{2+} which is independent of extracellular Ca^{2+} (Kao & Schneider, 1985) and mAChR stimulation of secretion occurs in the absence of extracellular Ca^{2+} (Nakazato, Yamada, Tomita & Ohga, 1984; Malhotra, Wakade & Wakade, 1988; Misbahuddin & Oka, 1988). Additional evidence indicates that the muscarine-induced elevation of cytosolic Ca^{2+} is associated with polyphosphoinositide breakdown (Eberhardt & Holz, 1987; Malhotra *et al.* 1988). Furthermore, inositol trisphosphate (IP_3) is known to trigger release of Ca^{2+} from intracellular stores in permeabilized chromaffin cells (Stoehr, Smolen, Holz & Agranoff, 1986). In sum, these results are consistent with the view that mAChR activation, presumably as a consequence of a G-protein-mediated activation of phospholipase C (PLC), produces the IP_3-mediated release of Ca^{2+} from intracellular stores.

To what extent the IP_3-mediated elevation of Ca_i^{2+} accounts entirely for the effects of muscarine remains unclear. In particular, prolonged application of muscarine to perfused rat adrenals supports sustained secretion of catecholamines (Warashina *et al.* 1989) at a time when the elevation of Ca_i^{2+} resulting from the activation of a PLC would be waning. We have been interested in the possibility that, in addition to its effects in promoting release of Ca^{2+} from intracellular stores, some effects of

muscarine may be mediated by modulation of particular components of membrane current. On the whole, the electrophysiological consequences of muscarine action on chromaffin cell function remain poorly defined. Earlier work on rat chromaffin cells has suggested that during prolonged mAChR receptor activation the action potential frequency is elevated (Brandt, Hagiwara, Kidokoro & Miyazaki, 1976; Kidokoro, Ritchie & Hagiwara, 1979). However, the basis for this effect has not been fully established. Recently, Akaike, Mine, Sasa & Takaori (1990*a*, *b*) have shown that in freshly isolated rat chromaffin cells muscarine can produce the inactivation of a K^+ current, which may account for the muscarine-induced elevation of action potential frequency. For comparison, sympathetic ganglionic neurones, which are homologous to chromaffin cells, express an M-current (I_M) and an after-hyperpolarization current (I_{AHP}), which are active at resting potentials and can play an important role in determining the pattern of electrical activity of those cells (Adams, Brown & Constanti, 1982*a*: Galvan & Sedlmeier, 1984; Pennefather, Jones, Adams & Nicoll, 1985; Goh & Pennefather, 1987). Muscarine is known to produce effects on both I_M (Adams, Brown & Constanti, 1982*b*) and I_{AHP} (Goh & Pennefather, 1987; Dutar & Nicoll, 1988). In the accompanying paper (Neely & Lingle, 1992), we have shown that rat chromaffin cells also exhibit a current similar to that responsible for I_{AHP}. Thus, rat chromaffin cells express at least one current which might be expected to regulate action potential frequency.

This paper presents an initial description of the basic features of muscarine action on outward current in rat chromaffin cells. The results indicate that the action of muscarine is quite complex both in the nature of the underlying conductance changes and in the temporal sequence of the changes. Some of the effects of muscarine are consistent with the activation of Ca^{2+}-dependent K^+ current resulting from a release of Ca^{2+} from intracellular stores. However, muscarine can also produce a transient inactivation of a K^+ current, presumably the voltage-dependent, Ca^{2+}-activated K^+ current. Some of these results have been presented in abbreviated form (Neely & Lingle, 1988, 1990).

METHODS

Cell preparation

Cells were prepared as described in the previous paper (Neely & Lingle, 1992) following previously developed procedures (Fenwick, Fajdiga, Howe & Livette, 1978; Kilpatrick, Ledbetter, Carson, Kirshner, Slepestis & Kirshner, 1980; Role & Perlman, 1980) except as follows. Cells used for measurements of cytosolic Ca^{2+} were grown for 2–9 days on Vitrogen-coated (Vitrogen 100, Collagen Corp., Palo Alto, CA, USA) glass coverslips placed in the bottom of 35 mm culture dishes.

Solutions

The standard intracellular solution contained the following: 120 mM-potassium aspartate; 30 mM-KCl; 2·1 mM-$MgCl_2$; 10·5 mM-HEPES (*N*-methylglucamine); 0·5 mM-EGTA; 2 mM-*myo*-inositol. This was supplemented with 2 mM-ATP (Na_2ATP), 0·5 mM-cAMP, 0·1 mM-leupeptin and 0·05 mM-GTP (Li_4GTP) and titrated to pH 7·4 with *N*-methylglucamine (NMG). The standard extracellular saline used for current recordings was the following: 120 mM-NaCl; 5·4 mM-KCl; 2 mM-$MgCl_2$; 1·8 mM-$CaCl_2$; 10 mM-HEPES (NMG); 20 mM-NMG-Cl, pH 7·4. Salines containing 20 mM-KCl, 1 or 5 mM-TEA or 5 mM-Co^{2+} were prepared by isosmotic substitution for NMG-Cl. The standard extracellular saline used for fluorescence measurements was identical except that 140 mM-NaCl was employed in place of 120 mM-NaCl/20 mM-NMG-Cl.

Electrophysiological methods and analysis

The basic features of the electrophysiological methods follow standard whole-cell recording methods (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) as described in the previous paper (Neely & Lingle, 1992) with the following exception. Voltage ramps were used in some experiments to examine the properties of currents modified by muscarine. A typical voltage ramp protocol was the following. From a holding potential of −49 mV, the membrane voltage was changed with an initial upward swing from −49 to +11 mV, and a downward swing to −109 mV, before returning to −49 mV. Except where noted, ramps were applied at a frequency of 0·5 Hz and the full ramp waveform lasted 1 s. Conductance at a particular potential during voltage ramps was calculated from the current at that potential divided by the difference between the ramp potential and the apparent reversal potential for the muscarine-activated current. All reported voltages include correction for a +9 mV liquid junction potential measured between the typical pipette saline containing potassium aspartate and the extracellular saline containing NaCl. Drug applications to voltage-clamped cells were accomplished either by a U-tube system or with a pipette jammed with up to seven perfusion lines, as described in the previous paper (Neely & Lingle, 1992).

Measurements of cytosolic Ca^{2+} transients

Cytosolic Ca^{2+} transients were examined in non-dialysed, non-voltage-clamped cells using the ratiometric measurement of Fura-2 fluorescence (Grynkiewicz, Poenie & Tsien, 1985). Glass coverslips with adherent chromaffin cells were removed from 35 mm culture dishes and clamped in a holder for mounting on the stage of a Zeiss IM-35 microscope modified for transmission of UV excitation light through the epifluorescence port. The cells were then incubated for 20–40 min at room temperature in a loading solution (140 mM-NaCl; 5 mM-KCl; 1·8 mM-$CaCl_2$; 2·0 mM-$MgCl_2$; 10 mM-HEPES; 5·6 mM-glucose) containing 2–4 μM-Fura-2 AM. In later experiments, the loading solution was mixed with an equal volume of the culture medium to yield a final Fura-2 AM concentration of 1–2 μM. Following cell loading, cells were then washed for 15–30 min in the standard extracellular saline used for eletrophysiological experiments prior to initiating Ca^{2+} measurements. Application of agents to non-dialysed cells was accomplished by the use of one or two puffer pipettes, which were heavily fire-polished patch micropipettes (1–5 μm tip diameter). The puffer pipettes were positioned 30–50 μm from the cell under investigation to minimize possible leakage of agonist onto the cell surface. For examination of the divalent cation sensitivity of muscarinic responses, the bath saline was exchanged and a second puffer pipette containing the appropriate concentration of agonist in the modified saline was then used to test the response to agonist.

A PTI (Princeton, NJ, USA) Deltascan dual-monochromator system was used for excitation of cells. In this system, light from a 75 W Xenon arc lamp is split by a spinning mirror and directed into two monochromators. For most experiments, excitation wavelengths were at 340 and 390 nm with a 5 nm bandpass. The two collimated light beams are recombined after exit from the monochromators and fluorescence emission is measured with a single photomultiplier with photon-counting electronics. For all experiments shown here fluorescence ratios were calculated at frequencies of 12–20 Hz. Uniform dispersion of the excitation light from both light paths at the level of the microscope stage was insured by proper positioning of a field lens in the epifluorescence path. Emitted light was limited to the area of a single cell by a pinhole aperture; with the 100× Nikon fluor oil-immersion objective used for these experiments, the aperture allowed sampling of an area with a diameter of about 25 μm. Background fluorescence of unloaded cells was less than 5% of the fluorescence of loaded cells and was ignored in the calculation of fluorescence ratios. The limitations of procedures for calibration of cells loaded via the permanent forms of the fluorescent Ca^{2+} buffers have been well documented (Almers & Neher, 1985; Benham, 1989). As such, all Ca^{2+} measurements are simply reported as the ratio of fluorescence stimulated by excitation at 340 nm to fluorescence stimulated by excitation at 390 nm.

Chemicals

Fura-2 AM was obtained from Molecular Probes. DL-Muscarine and other chemicals were from Sigma.

RESULTS

General characteristics of muscarine action on rat chromaffin cells

The application of muscarine to voltage-clamped rat chromaffin cells results in a complex set of conductance changes. The effects of muscarine to be considered in this paper are summarized in Fig. 1. Two basic procedures were used to examine the effects of muscarine on membrane currents: first, the effect of muscarine on cells clamped at a holding potential between −65 and −30 mV and, second, the effect of muscarine on currents elicited by a depolarizing and hyperpolarizing voltage ramp usually spanning the range of voltages from −109 to +11 mV.

An example of the first approach is illustrated in Fig. 1*A*. In a cell held at −49 mV, the application of 50 μM-muscarine leads to a rapid activation of outward current which declines even in the continuing presence of muscarine. The first portion of this paper focuses on the properties of this muscarine-activated transient current when recorded at holding potentials negative to −30 mV.

An example of the use of voltage ramps to examine the effects of muscarine on chromaffin cell membrane currents is shown in Fig. 1*B* while Fig. 1*C* illustrates a single voltage ramp and the resulting membrane current. In Fig. 1*B*, currents were elicited by the application of the voltage ramp waveform at 0·5 Hz, while muscarine was applied during the time indicated by the bar. In response to muscarine application, at the holding potential of −65 mV an outward current is observed. At the most negative voltage excursion (−115 mV), this outward current is reversed. At the most positive voltage excursion (+5 mV), an apparent inactivation of outward current is observed. The transient decrease in voltage-activated outward current observed at +5 mV in Fig. 1*B* occurs simultaneously or just subsequent to the activation of outward current observed at more negative potentials. Figure 1*D* illustrates the response of the same cell shown in Fig. 1*B* to muscarine application when the membrane voltage is simply stepped between −69 and −39 mV. At this display gain, the transient activation of outward current at potentials negative to −39 mV is emphasized.

The use of voltage ramps was necessary in order to assess possible effects of muscarinic receptor activation on voltage-dependent components of chromaffin cell membrane current. Although transient aspects of the muscarinic response cannot be considered to be fully at steady state during the ramps, the ramps allow a better indication of the temporal effects of muscarine action over a full range of membrane potentials than standard voltage step protocols. Thus, using the voltage ramp procedure, the second portion of this paper addresses the properties of the decrease in conductance produced by muscarine at more positive membrane potentials. Finally, a third feature of the action of muscarine observed in some cells is a prolonged conductance decrease which persists following removal of muscarine. In Figs 1*B* and 10*A*, this conductance decrease can be seen as a small decrease in current activated at the bottom excursion of the hyperpolarizing sweep of voltage. This phenomenon may simply represent run-down or persistent inactivation of Ca^{2+} currents and will not be considered further here.

Transient activation by muscarine of an outward current

At holding potentials negative to about −30 mV, muscarine at concentrations ranging from 0·5 to 50 μM was observed to activate an outward current, which after a concentration-dependent latency reaches a peak in less than 1 s. Figure 2*A* shows

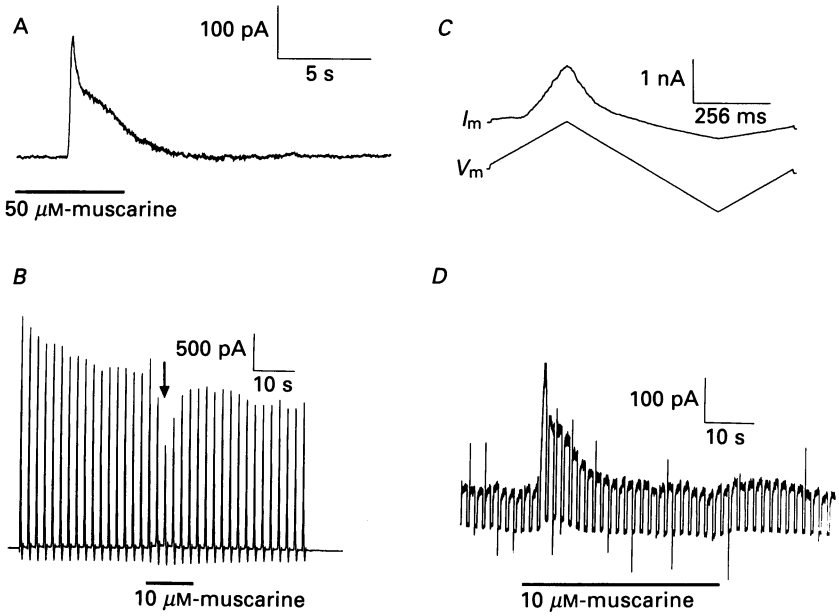

Fig. 1. Muscarine application results in multiple effects on membrane current in rat chromaffin cells. In *A*, the ability of 50 μM-muscarine to elicit a transient outward current in a cell held at −49 mV is shown. The period of muscarine application is indicated by the horizontal bar. Figure 10 illustrates the effect of 50 μM-muscarine on this same cell during activation of voltage-dependent currents by voltage ramps. In *B*, the effect of muscarine on currents elicited by repetitively applied voltage ramps in another cell is shown. At potentials negative to about −40 mV, muscarine activates a conductance increase while at the peak of the voltage ramp a conductance decrease is apparent. In this cell the holding potential was −65 mV, ramps were applied at a frequency of 0·5 Hz, and the ramp voltages ranged from −115 to +5 mV (in most other cases, the range was −109 to +11 mV). In *C*, the voltage ramp waveform used for most experiments and a typical resulting current are displayed. The particular ramp was taken from the sweep indicated by the arrow in *B*. The complete voltage ramp waveform was 1 s in duration. In *D*, the activation of outward current by muscarine is shown at higher gain for the same cell as shown in *B*. The membrane potential was repetitively stepped between −39 and −69 mV. Muscarine application resulted in a large conductance increase, with a reversal potential negative to −69 mV.

the current responses to application of 10 and 50 μM-muscarine applied to a cell clamped at −49 mV. A trace obtained in response to saline containing 20 mM-KCl is also shown to provide an indication of the rate of exchange of solution bathing the cell and shows that the activation of current by muscarine occurs with a clear lag following exposure of the cell to agonist. The higher concentration of muscarine results in a shift in response latency to an earlier time after agonist application. This

type of effect is consistent with models in which mAChR occupation is coupled to activation of current by a signalling process that involves generation of some intracellular messenger (e.g. Horn & Marty, 1988). The current typically lasts for several seconds, but exhibits inactivation even during the sustained application of

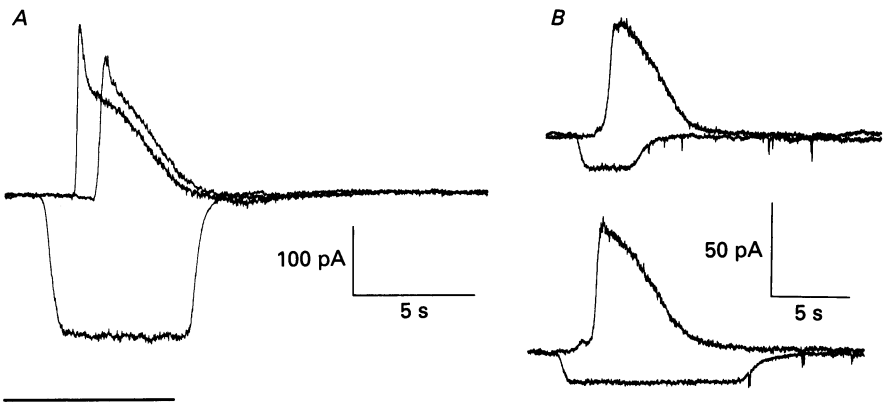

Fig. 2. Muscarine activates a transient outward current in single voltage-clamped rat chromaffin cells. In *A*, currents from a cell clamped at −49 mV are displayed. Traces correspond to currents in response to 10 μM-muscarine (smaller outward current), 50 μM-muscarine (larger outward current) or saline containing 20 mM-KCl (inward current). The current in response to elevated K^+ provides an indication of the rate of exchange of solution using the U-tube perfusion system. The horizontal bar indicates the time during which the solenoid controlling U-tube flow was closed. Increases in muscarine concentration produce only slight changes in outward current amplitude or time course, while producing clear decreases in response latency. Note that the difference between the time of complete exchange of solution around the cell and the activation of current by muscarine is hundreds of milliseconds to seconds. In *B*, the effect of a short and long application of 20 μM-muscarine to a second chromaffin cell clamped at −49 mV is shown. Inward current activated by saline with 20 mM-KCl provides an indication of the time course of drug application. The response amplitude and inactivation exhibit little difference with short or long applications of muscarine.

muscarine (Fig. 2 *B*). The shape of the outward current was quite similar among cells and was relatively independent of the concentration of agonist. However, some differences were noted in the shape of the decay of the transient current (compare Figs 2 and 4–6). The decay of outward current in some cases (Fig. 2*A*) appeared to exhibit a rather complex multiphasic decay, while in other cases the current turned off in a more exponential fashion (Fig. 2*B*). The former type of waveform could indicate that the apparent turn-off of outward current may, in part, reflect activation of an opposing inward current. Under the ionic conditions of the experiment shown in Fig. 2*A*, a non-specific cation conductance would be the most likely candidate for such a current (Colquhoun, Neher, Reuter & Stevens, 1981; Yellen, 1982; Marty, Tan & Trautmann, 1984) and, as will be shown below, the activation of such a current would probably be dependent on Ca^{2+}. However, in our examination of Ca^{2+}-activated current in these cells (Neely & Lingle, 1992), we have been unable to identify any Ca^{2+}-activated current other than two distinct Ca^{2+}-activated K^+ currents. An alternative possibility is that the outward current can, in

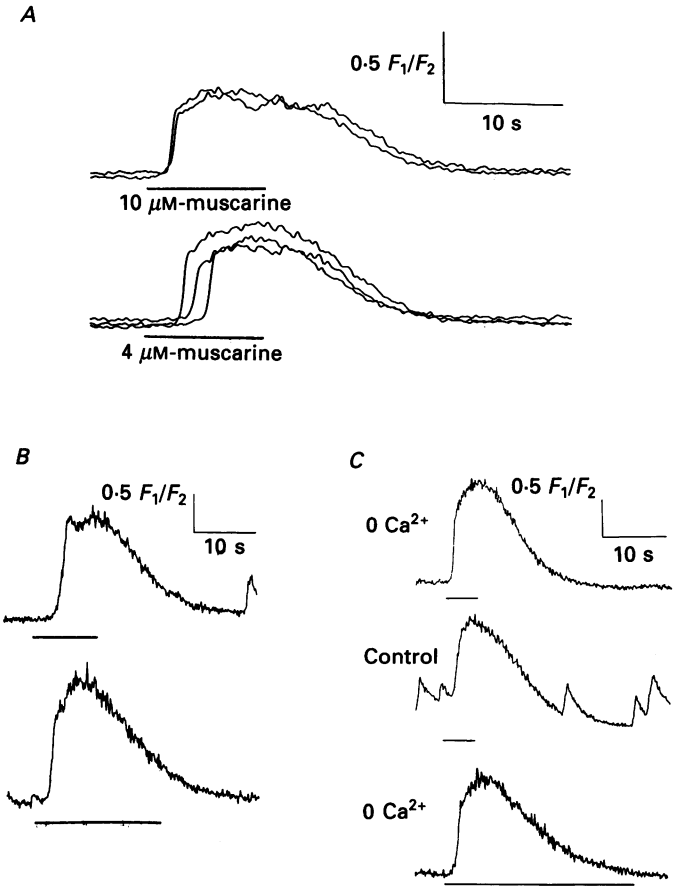

Fig. 3. Muscarine produces a transient increase in cytosolic Ca^{2+} in single rat chromaffin cells. In *A*, fluorescence ratio signals in response to puffs of 4 and 10 μM-muscarine onto a single non-dialysed chromaffin cell are illustrated. The top pair of traces show responses to two separate applications of 10 μM-muscarine, while the bottom traces show responses to three separate applications of 4 μM-muscarine. Increases in muscarine concentration decrease the time to maximum rate of rise of cytosolic Ca^{2+} without significantly altering the shape of the response. Response latencies typically exhibit more variability at muscarine concentrations of 4 μM and lower. In *B*, 10 μM-muscarine was applied to a single chromaffin cell for 10 s (top trace) and 20 s (bottom trace). Cytosolic Ca^{2+} levels return to control levels even during the sustained application of muscarine. In *C*, 12 μM-muscarine was applied to a single chromaffin cell in each trace. On the top and bottom, the cell was bathed in 0 mM-Ca^{2+} saline and, in the middle, the cell was bathed in control saline (1·8 mM-Ca^{2+}), Muscarine (in 0 mM-Ca^{2+} saline) was applied for 5 s for the top and middle traces and for 30 s for the bottom trace. The presence or absence of extracellular Ca^{2+} does not influence the duration of the Ca^{2+} elevation. The spontaneous Ca^{2+} transients in the middle trace presumably represent the elevation of Ca^{2+} resulting from single chromaffin cell action potentials. These transients are abolished in 0 Ca^{2+} saline or in saline containing 2 mM-Co^{2+}. Vertical calibration bars refer to the ratio of fluorescence (F_1/F_2) measured at two alternating excitation wavelengths.

some cases, consist of two components, each of which activates and turns off with a distinct time course.

Muscarine-induced changes in cytosolic Ca^{2+}

The transient activation of outward current by muscarine is reminiscent of effects of muscarine and other secretagogues on a number of secretory cell types (Ozawa, 1981; Trautmann & Marty, 1984; Mollard, Vacher, Dufy, Winiger & Schlegel, 1988; Gray, 1988; Horn & Marty, 1988; Maruyama, 1989), and there has also been an initial report of a similar phenomenon in rat chromaffin cells (Kubo & Kidokoro, 1989). In many cases, those effects are known to result from the secretagogue-induced elevation of cytosolic Ca^{2+}, resulting from release of Ca^{2+} from intracellular stores. A voltage-independent, Ca^{2+}-activated current is, in fact, a major contributor to outward current in rat chromaffin cells (Neely & Lingle, 1992) and is a reasonable candidate for a current that might underlie this effect of muscarine. Thus, the effect of muscarine on Ca_i^{2+} in single non-dialysed, unclamped chromaffin cells was studied using measurements of Fura-2 fluorescence to determine whether changes in cytosolic Ca^{2+} might participate in the muscarinic response.

As judged by spontaneous action currents measured with cell-attached patches, cultured rat chromaffin cells exhibit a range of electrical behaviours. Some cells are relatively quiescent and require mild patch depolarization or hyperpolarization to elicit action potentials. Other cells exhibit rather chaotic firing patterns and, in some cases, action potentials may be grouped in bursts. In previous work, rat chromaffin cells were observed to fire action potentials at frequencies of about 0·05–0·1 Hz and, in the presence of TEA, ACh or elevated K_o^+, action potential frequency was increased up to about 0·5–2·0 Hz (Brandt *et al.* 1976; Kidokoro *et al.* 1979; Kidokoro & Ritchie, 1980). Such spontaneous electrical activity, which can be quite complex, is responsible for the generation of spontaneous Ca^{2+} transients observed in many rat chromaffin cells. The properties of the underlying spontaneous activity are considered in more detail below (see Figs 4 and 12). Such spontaneous Ca^{2+} signals can complicate the interpretation of the effects of muscarine. To illustrate some of the basic features of the time course and recovery of the response to muscarine, the results shown in Fig. 3 were selected from relatively quiescent cells. However, no differences in the sensitivity or basic properties of the response to muscarine have been associated with the intrinsic activity of the cells.

Figure 3*A* shows that, in a non-dialysed chromaffin cell loaded with Fura-2, muscarine elicits a transient increase in cytosolic Ca^{2+}. The latency to maximum rate of rise of the muscarine-induced elevation of Ca^{2+} exhibited a dependence on agonist (Fig. 3*A*) similar to the muscarine-activated outward current. At concentrations of 4 μM or below, the latency exhibited considerably more variability among muscarine applications as seen in Fig. 3*A*. Also, similar to the properties of the activation of outward current by muscarine, the muscarine-induced elevation of Ca^{2+} was transient even in the sustained presence of muscarine (Fig. 3*B* and *C*). In Fig. 3*B*, the Ca^{2+} elevations in response to 10 and 20 s applications of 10 μM-muscarine are shown. The durations of the two responses are similar and the elevation of Ca^{2+} has returned almost to resting levels at the end of the 20 s application.

The origin of the elevation of cytosolic Ca^{2+} involved in the muscarinic effect was

addressed in Fig. 3*C*. On the top, the effect of extracellular saline with nominally 0 Ca^{2+} on the time course of muscarine-induced elevations of Ca^{2+} is shown. The top and bottom traces illustrate responses to 5 and 30 s applications of 12 μM-muscarinic in 0 Ca^{2+} saline. The middle trace illustrates an interposed response to a 5 s application of 12 μM-muscarine in saline with 1·8 mM-Ca^{2+}. In contrast to the traces obtained in 0 Ca^{2+} saline, transient elevations of Ca^{2+} resulting from presumed spontaneous action potentials occur together with the mAChR-induced elevation of Ca^{2+}. The similarity of the time course of each response in the presence and absence of Ca^{2+} indicates that the time course of the response is not significantly influenced by the influx of Ca^{2+}. For both short and long applications of muscarine, there was little change in the shape of the Ca^{2+} elevation. The experiment indicates that, at a time when influx of Ca^{2+} resulting from spontaneous action potentials is not observed, muscarine can still produce a transient elevation of cytosolic Ca^{2+}. In other experiments (not shown) the application of saline containing 2 mM-cobalt blocked Ca^{2+} transients resulting from spontaneous action potentials but was without effect on muscarine-induced Ca^{2+} transients. Exposure of cells to 2 mM-cobalt or 0 Ca^{2+} saline for periods longer than about 3 min resulted in a gradual diminution of the muscarine-induced Ca^{2+} transient, presumably as a result of depletion of Ca^{2+} available for refilling of intracellular stores. These results support the view that mAChR activation results in an elevation of cytosolic Ca^{2+} by a mechanism that does not require Ca^{2+} influx.

In a number of cells, it was observed that, during prolonged applications of muscarine, following an initial transient elevation of Ca^{2+}, there was a more sustained elevation of Ca^{2+} which persisted during the application of muscarine (e.g. Fig. 12*A*). The sustained elevations were typically associated with large and periodic noise in the fluorescence ratio signal suggestive of increased action potential frequency. In such cells, the application of muscarine, either in the presence of 2 mM-Co^{2+} or with 0 Ca^{2+} saline, results solely in a transient elevation of cytosolic Ca^{2+}. Tentatively, we attribute sustained elevations to effects of mAChR activation that lead to increased action potential frequency that occur subsequent to the transient release of Ca^{2+} from intracellular stores. Additional experiments are required to resolve this point.

A comparison (e.g. Figs 2 and 3) of the time course of the mAChR-induced cytosolic Ca^{2+} transients and of the activation of outward current shows that the duration of cytosolic Ca^{2+} transient is consistently longer than the muscarine-activated outward current. In the absence of simultaneous current and fluorescence measurements, the significance of this difference is unclear.

For comparison to the effect of muscarine, changes in cytosolic Ca^{2+} occurring spontaneously and in response to small elevations in KCl are shown in Fig. 4. In the absence of direct simultaneous electrical measurements, we interpret the spontaneous activity as the result of Ca^{2+} influx during action potentials. This is based on the following considerations. First, the spontaneous transients (Fig. 4*A*) are similar in time course to transients initiated by moderate KCl (15 mM) (Fig. 4*B*) and nicotine (10 μM) concentrations. Second, the spontaneous transients are rapidly abolished by 0 Ca^{2+} saline (Fig. 3*C*) or the addition of 2 mM-cobalt to the saline. Third, the frequencies of occurrence of these transients and the increase in frequency stimulated

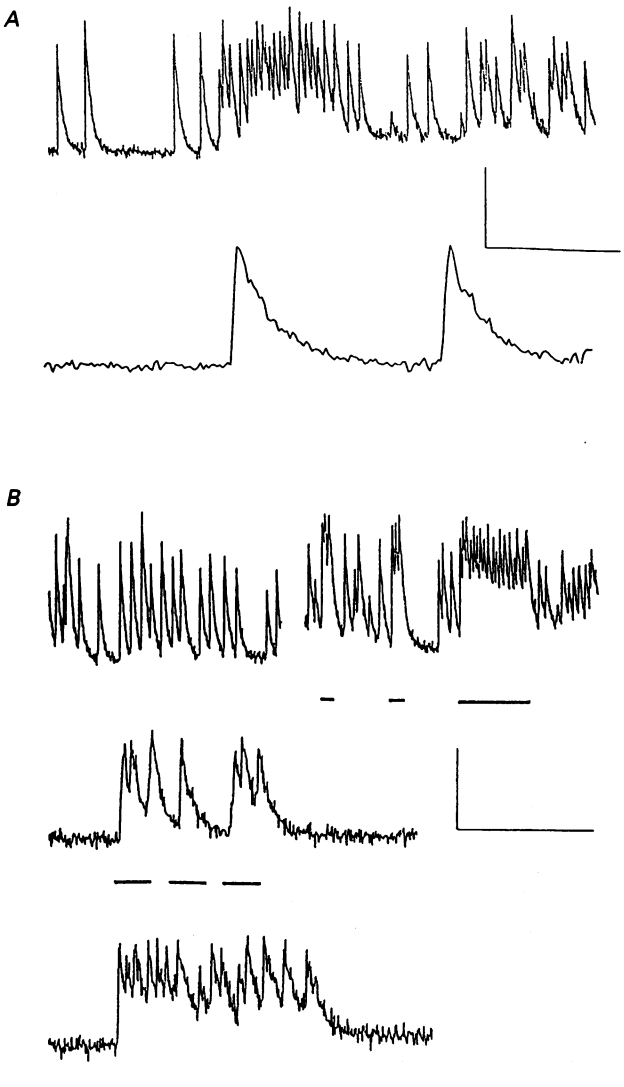

Fig. 4. Spontaneous and KCl-induced Ca^{2+} transients. In *A*, spontaneous Ca^{2+} transients observed in a single rat chromaffin cell are displayed in the top trace. Transients occur randomly or in poorly defined bursts. The bottom trace shows individual Ca^{2+} transients at a more rapid time base. The Ca^{2+} transients peak in 200–400 ms and decay over about 10 s. The fluorescence ratios were calculated at a rate of 12 Hz. Vertical calibration: 0·5 F_{340}/F_{390}; horizontal calibration: top trace, 80 s; bottom trace, 10 s. In *B*, the effect of 15 mM-KCl (applied during the bars) on spontaneous Ca^{2+} transients is illustrated. KCl increases the frequency of spontaneous transients in the top trace. The pipette containing saline with 15 mM-KCl was lowered during the gap in the top trace. The bottom two traces illustrate the effect of 15 mM-KCl applied to a quiescent cell. This concentration of KCl results in the occurrence of repetitive spontaneous Ca^{2+} transients. Vertical calibration: 0·5 F_{340}/F_{390}; horizontal calibration: top trace, 80 s; bottom two traces, 20 s.

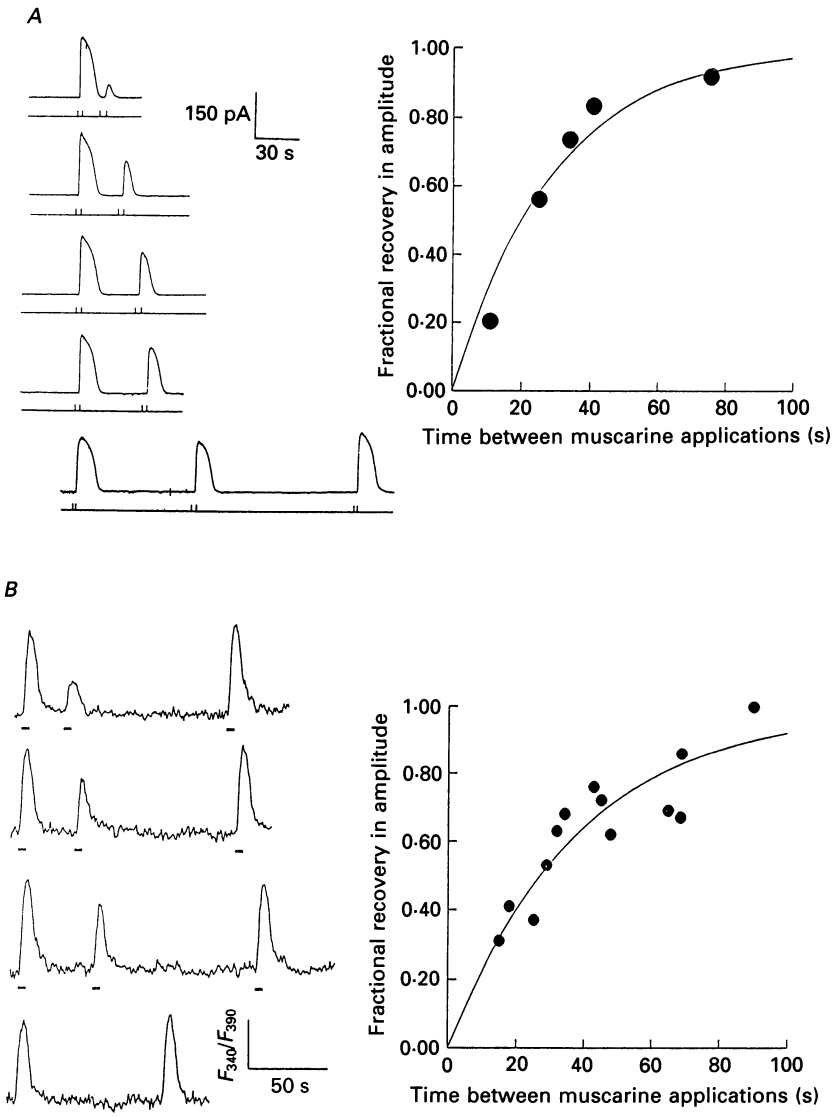

Fig. 5. Recovery of muscarine-activated responses. In *A*, a chromaffin cell clamped at −49 mV was exposed to pairs of ~ 5 s applications of 40 μM-muscarine. On the left, examples of outward currents activated by muscarine are shown. Muscarine was applied for the periods indicated by the vertical tick marks on the horizontal line below each current trace. On the right the fractional recovery between pulse pairs is plotted as a function of pulse separation. The line over the data points is a single exponential fit with time constant of 29 s. In *B*, the cytosolic Ca^{2+} transients measured as fluorescence ratios were elicited by pairs of 5 s puffs of 20 μM-muscarine in a non-dialysed, non-clamped cell. On the left, muscarine was applied during the periods indicated by the horizontal bar. On the right, the fractional recovery in amplitude for the response to the second application of muscarine is plotted as a function of time between muscarine applications. Points were obtained from three cells. The line is a single exponential fit with time constant of 38 s.

by KCl (Fig. 4*B*) and nicotine are similar to the behaviour of extracellularly recorded spontaneous action potentials in rat chromaffin cells (Brandt *et al.* 1976; Kidokoro *et al.* 1979; Kidokoro & Ritchie, 1980). In some cells, the frequency of spontaneous activity was sufficiently high that it was not possible to distinguish the contribution of Ca^{2+} elevation due to individual action potentials. In such cases, the fluorescence signal exhibited increased noise compared to quiescent cells (e.g. see Fig. 12*D*).

Both 10 μM-nicotine and 15 mM-KCl produced an increase in Ca^{2+} transient frequency in active cells or induced Ca^{2+} transients in quiescent cells. In both of these cases, the transients presumably reflect action potential activity and, hence, the transients provide a measure of the rate of clearance of Ca^{2+} following pulsatile influx of Ca^{2+} through membrane Ca^{2+} channels. For present purposes it is notable that the rise time of such transients is hundreds of milliseconds in duration and Ca^{2+} remains elevated for almost 10 s (Fig. 4 *A*). These values are similar to the rise and decay of Ca_i^{2+} resulting from spontaneous action potentials (Mollard *et al.* 1988) and voltage-gated Ca^{2+} influx (Benham, 1989) in GH_3 clonal pituitary cells. In contrast to this relatively slow clearance of Ca^{2+} as measured over the whole chromaffin cell, tail current resulting from activation of a voltage-independent Ca^{2+}-activated K^+ current following brief (< 100 ms) voltage steps to $+20$ mV under probably not too dissimilar buffering conditions (0·5 mM-EGTA *versus* a concentration of Fura-2 probably less than 300 μM) is complete in milliseconds (Neely & Lingle, 1992). The slow time course of Ca^{2+} transients resulting from relatively pulsatile influx of Ca^{2+} again suggests a discrepancy between the fluorescence estimate of Ca_i^{2+} changes and the time course of submembrane Ca^{2+} indicated by Ca^{2+}-activated K^+ currents.

The time course of recovery and stability of the muscarine-activated outward current

The muscarine-activated outward current peaks and terminates prior to completion of the application of muscarine. The transient nature of this current is not likely to be the result of an intrinsic time-dependent inactivation of the current itself, since as shown below the current underlying this response appears to result primarily from a non-inactivating, voltage-independent, Ca^{2+}-dependent K^+ current. Rather, some step in the sequence linking muscarinic receptor occupation to outward current flow appears to undergo desensitization or depletion. The approximate time course of recovery from this inactivation process was assessed by an examination of the recovery in the response between paired muscarine pulses. As shown in Fig. 5*A* for a cell clamped at -49 mV, the time between pairs of applications of 40 μM-muscarine was varied between about 15 s and almost 80 s. At times near 2 min (bottom trace in Fig. 5*A*), almost complete recovery of the ability of muscarine to activate outward current is observed, while at shorter intervals between applications of muscarine the second response is markedly reduced. For this cell, clamped at -49 mV, a single exponential fit to the recovery time course yielded a time constant of 29 ms.

Similarly, the recovery of the ability of muscarine to elicit elevations of cytosolic Ca^{2+} occurred over a 1–2 min period (Fig. 5*B*). Pairs of 5 s applications of 20 μM-muscarine were applied to a non-dialysed chromaffin cell yielding the fluorescence ratios seen on the left. On the right, fractional recoveries from similar pulse pairs from three cells are plotted. A single exponential fit to the recovery time course yields

a time constant of 38 s. Whether measured from the increase in cytosolic Ca^{2+} or from the activation of outward current, recovery in the ability of muscarine to elicit a response follows a remarkably similar time course.

One other feature of the inactivation phenomenon is that reduced responses, whether measured with membrane current or as changes in Ca_i^{2+}, also exhibit

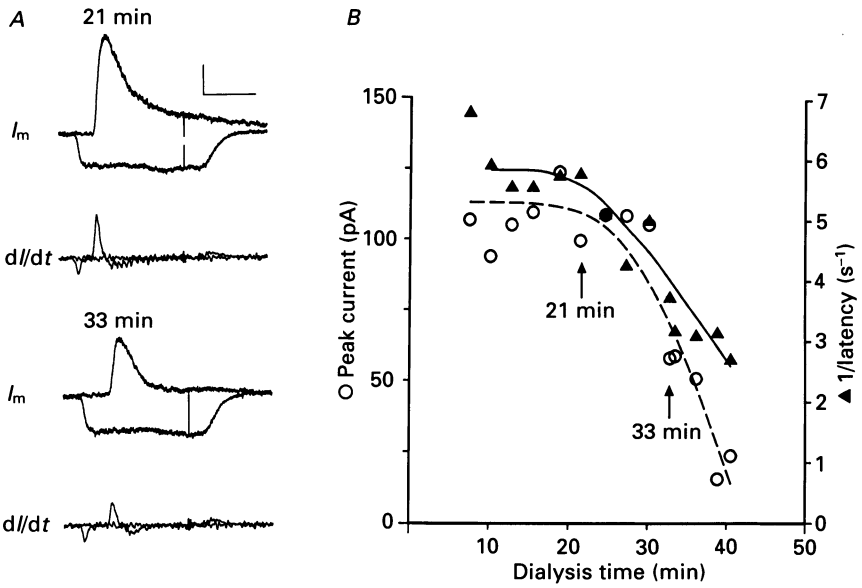

Fig. 6. Time course of run-down of muscarine-activated responses in dialysed cells. In *A*, examples of outward currents elicited by application of 20 μM-muscarine to a voltage-clamped cell are displayed for responses at 21 min (top trace) and 33 min (third trace) after initiation of whole-cell recording. The inward current provides a measure of the solution exchange time in each case. The second and fourth traces (labelled dI/dt) in *A* are differentiated traces of the sweeps shown in the first and third traces in order to illustrate the time of maximum rate of current rise. The time between maximum rate of current increase in response to application of saline with 20 mM-KCl and the maximum rate of rise of the muscarine-evoked outward current provides a measure of the intrinsic latency between receptor occupation and response onset. Muscarine was applied every 2–3 min. Over a 20–30 min period little change in response amplitude, latency or time course is observed. Membrane conductance (C_m), 7 pF; series resistance (R), 5·95 MΩ. Vertical calibration, 25 pA; horizontal calibration, 2·5 s. In *B*, the amplitude and latencies of the responses for the cell shown in *A* are plotted over time. The triangles correspond to the latency to maximal rate of activation of the current while the open circles correspond to the peak current amplitude. The lines are simple hyperbolic functions drawn to illustrate the overall trends with no mechanistic implications. Labelled arrows indicate the time of the responses shown in *A*.

markedly longer latencies to the maximal rate of rise of the response. However, when an adequate recovery time is allowed, both amplitude and latency are restored.

If intervals between muscarine applications are limited to times greater than about 1 min, muscarine-activated outward current shows only a small decrement in amplitude or increase in latency over a period of 20–30 min (Fig. 6). This suggests that the factors influencing the time course of recovery between muscarine pulses

and the stability of the responses are reasonably well maintained by the composition of the intracellular saline used in these experiments. At present, we have no information about which component(s), if any, of our standard intracellular saline might be important in the maintenance of the muscarine-activated outward current response in chromaffin cells. The stability of the muscarinic response under our experimental conditions appears to differ from a similar muscarinic response in lacrimal gland cells (Horn & Marty, 1988).

The stability of the muscarine-activated current response in dialysed cells is similar to the stability of muscarine-activated increases in intracellular Ca^{2+} in non-dialysed cells. Muscarine-induced elevations of cytosolic Ca^{2+} measured by Fura-2 fluorescence can be elicited for periods of at least 30 min. However, some gradual diminution in the ability of muscarine to produce an elevation of Ca^{2+} can occur even when 2–3 min of recovery is allowed between muscarine applications (e.g. Fig. 12*D*). The fact that some run-down in the ability of muscarine to elevate cytosolic Ca^{2+} occurs even in non-dialysed cells suggests that some slow desensitization of muscarinic sensitivity may occur even in the absence of cellular dialysis.

The results presented to this point indicate that muscarinic results in an elevation in free cytosolic Ca^{2+} and an activation of outward current. In a previous paper (Neely & Lingle, 1992), it was shown that elevation of cytosolic Ca^{2+} in chromaffin cells can result in activation of two distinct components of Ca^{2+}-dependent K^+ current. Based on similarities of these two currents to Ca^{2+}-dependent currents in other cell types, it is convenient to refer to the currents as the SK current (Blatz & Magleby, 1986; Ritchie, 1987*a*: Lang & Ritchie, 1987) and BK current (Marty, 1981; Barrett, Magleby & Pallotta, 1982; Magleby & Pallotta, 1983; Yellen, 1984; Marty & Neher, 1985), indicative, respectively, of the voltage-independent and voltage-dependent components of Ca^{2+}-activated current. The following experiments provide pharmacological tests of the identity of muscarine-activated outward current in two ways: first, by examining the properties of transient outward current at a fixed holding potential and, second, by the use of voltage ramps.

Pharmacological properties of muscarine-activated outward current at a fixed holding potential

To address the possibility that SK-like current underlies the muscarine-induced outward current at relatively negative holding potentials, Fig. 7*A* illustrates the effect of curare on the muscarine-activated transient outward current. Activation of outward current by application of muscarine at holding potentials more negative than about −40 mV was blocked almost completely by 200 μM-curare. The slight residual current activated by muscarine in the presence of curare may represent either curare-resistant current or the time course of blockade by curare.

A comparison of the effect of 200 nM-apamin and 1 mM-TEA on transient outward current activated by muscarine at −49 mV is shown in Fig. 7*B*. One millimolar TEA reduced the transient outward current about 10–15% while apamin blocked over 80% of the current. This result is consistent with most of the current activated at potentials negative to −40 mV arising from apamin-sensitive channels. The blockade by TEA is, in part, consistent with the expected amount of blockade of SK current by TEA based on the overlap between TEA and apamin block of Ca^{2+}-dependent

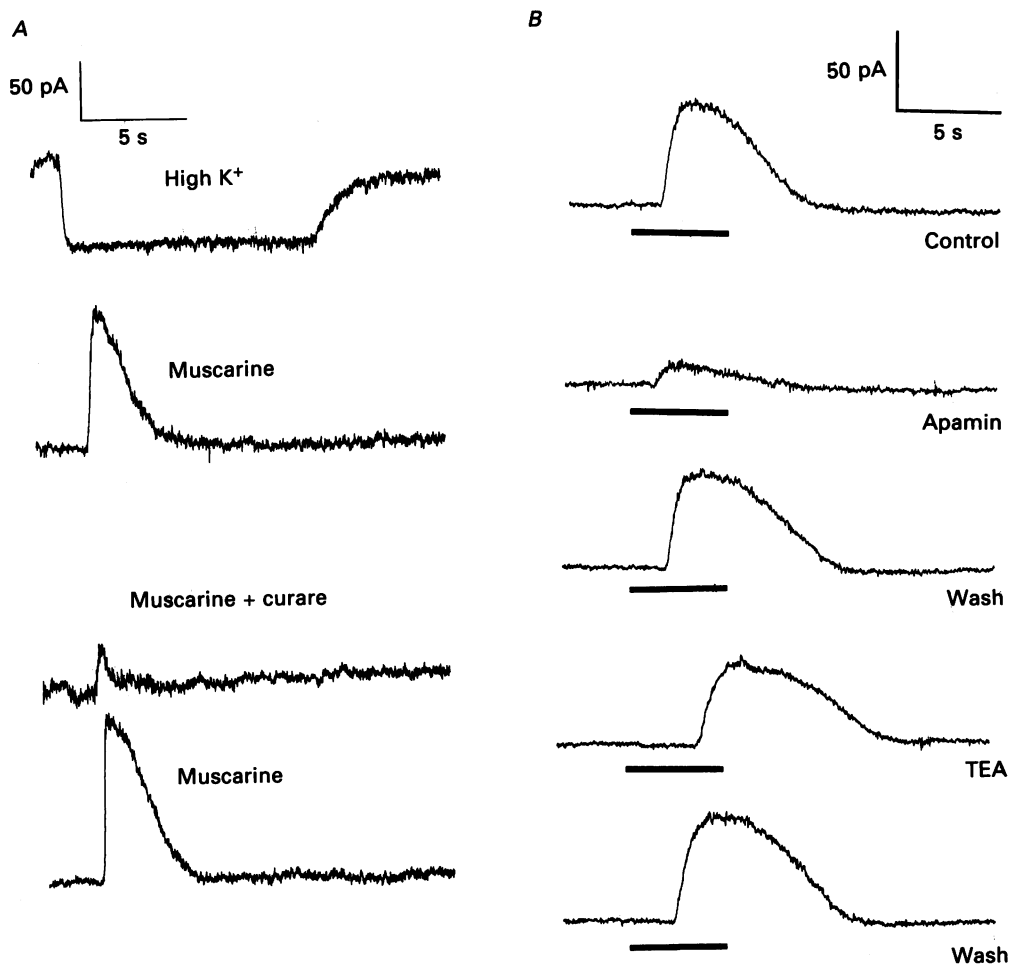

Fig. 7. Muscarine-activated outward current is inhibited by apamin and curare. In *A*, the sensitivity of muscarine-activated transient outward current is illustrated. Currents were recorded in a cell clamped at −49 mV. The top trace illustrates currents activated by an application of elevated potassium saline (20·4 mM-K^+). The subsequent traces show, from top to bottom, responses to 20 μM-muscarine, 20 μM-muscarine plus 200 μM-curare, and the recovery to 20 μM-muscarine. Drug applications were via U-tube perfusion and the cell was not pre-exposed to curare in the third trace. In *B*, the sensitivity of muscarine-activated transient outward current to apamin and TEA is illustrated. The top, middle and bottom traces illustrate responses to 40 μM-muscarine in control saline. The second trace shows the effect of 200 nM-apamin on the muscarine-activated current. The response is reduced by about 80%. The fourth trace shows that 1 mM-TEA reduced the response by about 20%. Note the shift in response latency in 1 mM-TEA and some recovery in latency on the bottom. Solution application was via a multibarrelled system (see Neely & Lingle, 1992). In the second and fourth traces, cells were pre-exposed to TEA and apamin, respectively, before application of muscarine in the presence of the blocking drug.

slow tail current (Neely & Lingle, 1992). However, in other cells in which apamin or curare also blocked over 80% of the muscarine-activated outward current, 1 mM-TEA was observed to block as much as 50% of the current. At present, we have no

explanation for the variability in the effect of TEA on the muscarine-induced activation of outward current (consisting primarily of apamin-sensitive current).

Irrespective of the explanation of the effects of TEA, Fig. 7 supports the view that at voltages more negative than about −40 mV muscarine results primarily in the

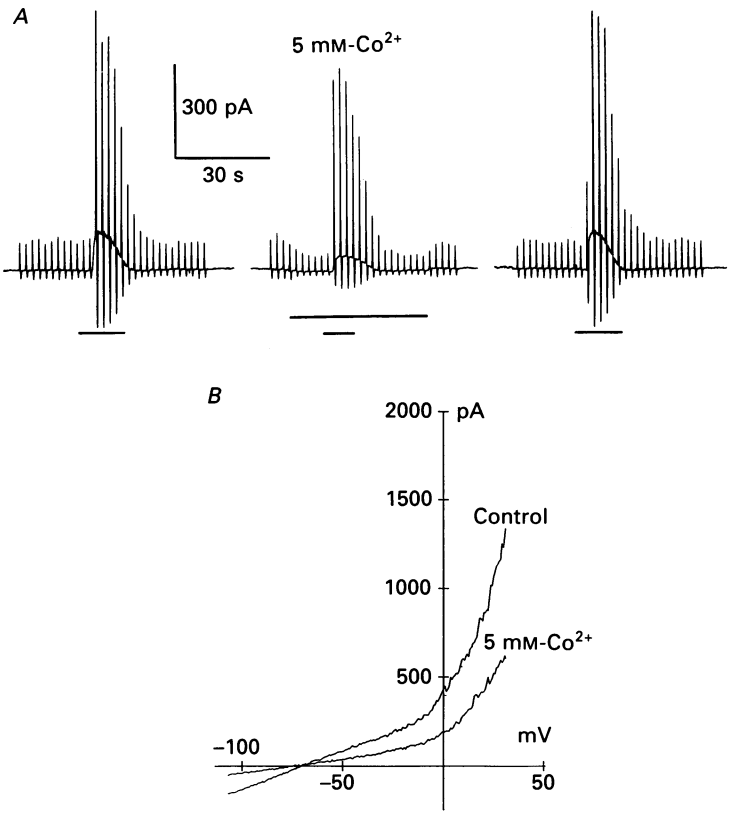

Fig. 8. Muscarine activates outward current in the presence of Co^{2+}. In *A*, the traces show continuous recordings of currents elicited by voltage ramps and the activation of currents by 40 μM-muscarine during those ramps. The left-hand trace was obtained with control saline, the middle trace with 5 mM-Co^{2+}, and the right-hand trace after return to normal saline. The voltage ramp waveform was similar to that in Fig. 1*C* but spanned potentials from −109 to +31 mV. The period of muscarine application is indicated by the bar below each trace. The longer bar in the middle trace corresponds to the period of 5 mM-Co^{2+} application. In *B*, the muscarine-activated current both in control saline and in the presence of 5 mM-Co^{2+} is plotted as a function of voltage. The muscarine-activated current was obtained from the difference between the current from a ramp at the peak of the muscarine response and the current during a ramp just prior to muscarine application. Co^{2+} results in some reduction of the total current activated by muscarine both at more negative and positive voltages. This may, in part, reflect a reduction of total cytosolic Ca^{2+} accumulation due to the blockade of Ca^{2+} influx during the voltage ramps. Note the reduction in outward current at both positive and negative sweeps of the voltage ramp in the presence of Co^{2+} in *A*, indicating that a significant amount of the outward current activated during the ramps results from Ca^{2+} influx. In other cells, 5 mM-Co^{2+} was without detectable effect on the muscarine-activated outward current.

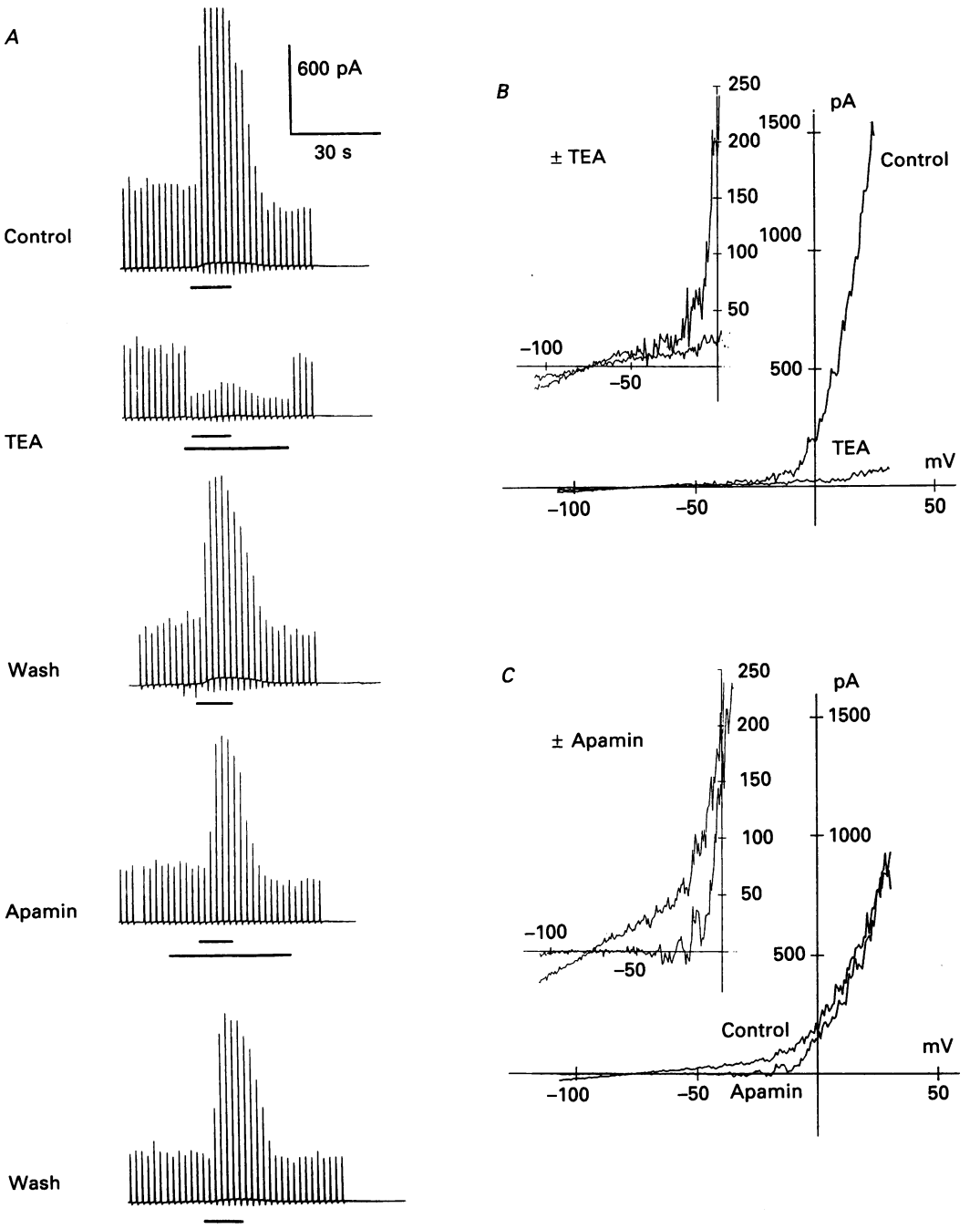

Fig. 9. Effects of apamin and TEA on muscarine-activated currents observed during voltage ramps. In *A*, examples of currents elicited during repetitive application of voltage ramps are shown. For each sweep, 40 μM-muscarine was applied during the period indicated by the bar. The top, middle and bottom traces show responses in normal saline. The peak currents during muscarine application in the top trace were truncated for display purposes. During muscarine application, there is an increase in current both at the most positive and negative excursions of the ramp (ramps spanned -109 to $+31$ mV). In

activation of an apamin- and curare-sensitive, but relatively TEA-resistant, current. This result is consistent with the idea that muscarine-induced elevations of cytosolic Ca^{2+} result in activation of the SK-like current found in these cells (Neely & Lingle, 1992).

Properties of muscarine-activated outward current activated by voltage ramps

The transient nature of the muscarine-activated response limits the utility of voltage jumps for analysis of effects on voltage-dependent current. As an alternative, currents were elicited by 1 s voltage ramps as shown in Fig. 1*C*. Muscarine can result in either an activation or inactivation of outward current at positive potentials. We will first focus on the activation of current prior to addressing the inactivation phenomenon.

In cells which exhibit activation of outward current at positive ramp potentials, an upward curvature characteristic of the activation of voltage-dependent current is seen at more positive voltages (e.g. Figs 8 and 9). Figure 8 demonstrates that the muscarinic response as measured with the voltage ramp method persists in the presence of 5 mM-Co^{2+}. In Fig. 8*A*, continuous records of currents elicited by voltage ramps and the response to a 5 s application of muscarine are shown for control saline, 5 mM-Co^{2+}, and after wash. In this cell there was some reduction in the current activated during the presence of muscarine, while in other cells no reduction was observed. Some reduction would, in part be expected from the reduction of total Ca^{2+} accumulation during the blockade of Ca^{2+} channels and a resulting more efficient clearance of Ca^{2+} at lower cytosolic Ca^{2+} loads. The occurrence of the muscarine response in the presence of Co^{2+} is consistent with earlier results showing that activation of outward currents initiated by muscarine occurs in the absence of Ca^{2+} influx. In Fig. 8*B*, the difference currents between voltage ramps in the presence and absence of muscarine are plotted both for the sets of ramps in the control saline and in the presence of Co^{2+}. The two lines plot, respectively, the current activated by muscarine either in the absence or presence of Co^{2+}. The shape of the muscarine-activated current in both cases suggests that at more positive voltages a portion of the current activated by muscarine involves the Ca^{2+}- and voltage-activated, TEA-sensitive BK-like current of rat chromaffin cells, while at more negative potentials a voltage-independent component of current is activated.

Figure 9 examines the pharmacological sensitivity of currents activated by muscarine in response to voltage ramps. Currents were elicited by 1 s voltage ramps

---

the second sweep, the muscarinic response was evoked in the presence of 1 mM-TEA which resulted in profound inhibition of current at positive potentials and weaker block near the holding potential. In the fourth trace, the effect of 200 nM-apamin is shown. Apamin results in almost complete block of current near the holding potential. In *B*, the muscarine-activated current (calculated as the difference between ramps at the peak of the muscarine response and just prior to muscarine application) is plotted as a function of membrane potential for currents obtained either in the absence or presence of 1 mM-TEA. The large effect of TEA on current positive to −40 mV is clearly shown. The inset replots the same data at higher resolution over the range of −100 to 0 mV and shows weaker blocking effects of TEA at more negative potentials. In *C*, muscarine-activated current is plotted for sweeps obtained either in the absence or presence of 200 nM-apamin. The inset shows the same current–voltage relation at higher gain. Apamin blocks all current negative to −40 mV.

applied at 0·5 Hz before, during and after application of muscarine. The muscarine-sensitive current was then obtained as in Fig. 8*B*. Ramps generated from a holding potential of −49 mV show that increases in conductance can be observed over both more negative and more positive voltages. Over the range of −109 to about −50 mV, the muscarine-activated conductance increase is predominantly linear. At potentials above about −40 mV, an increase in conductance with voltage is observed. One millimolar TEA blocks current at both extremes of the voltage ramp (Fig. 9*A*, second record). The muscarine-activated current both in the presence and absence of TEA is plotted in Fig. 9*B*. TEA blocks virtually all of the voltage-dependent component of muscarine-activated outward current, while having smaller inhibitory effects on the voltage-independent component. In contrast, 200 nM-apamin results in blockade of most current activated by muscarine at more negative potentials (Fig. 9*A*, fourth record). The effect of apamin on the muscarine-activated current is plotted in Fig. 9*C*. Apamin blocks almost all current negative to about −50 mV, while having no observable effect on the voltage-dependent component of current activated by muscarine. In separate experiments, 200 μM-curare was shown to have effects similar to apamin although the blocking effect of curare on currents negative to about −40 mV appeared to be less complete.

Comparison of conductance increases and decreases

The results presented above have focused on the basis of muscarine-induced activation of outward current. The results support the view that a muscarine-induced elevation of cytosolic Ca^{2+} can activate two previously described components of Ca^{2+}-dependent K^+ current in these cells. However, as indicated earlier, analysis of the voltage ramp-evoked currents shows that muscarine can also produce a transient conductance decrease in rat chromaffin cells. Our present results provide only a partial description of this effect.

Figure 10 illustrates properties of the transient conductance decrease using the ramp procedure utilized in previous experiments. During the application of 50 μM-muscarine to the cell the typical transient outward current is observed at the holding potential of −49 mV and a similar conductance increase at more negative potentials. However, at the most positive potentials, a dramatic decrease in outward current is apparent. In Fig. 10*B*, the chord conductances calculated at +6 and at −54 mV are plotted for each voltage ramp. During the action of muscarine the chord conductance at +6 mV falls to a value comparable to that observed at −54 mV consistent with the view that there is no additional current activated at +6 mV that is not already activated at −54 mV. Figure 10*C* shows the current–voltage relationship for current in response to a control voltage ramp and a ramp at the peak of the muscarine effect. Again, the removal of voltage-activated current by muscarine is apparent. In this cell, virtually all current present at the peak of the response to muscarine is voltage independent. As shown in the previous paper (Neely & Lingle, 1992), at +6 mV most voltage-dependent outward current in the rat chromaffin cells is Ca^{2+}-dependent current. The linearity of the current–voltage relationship during the peak of the inactivation suggests that the current which undergoes inactivation is the Ca^{2+}- and voltage-activated BK current. The similarity in the time course of inactivation of outward current at positive potentials with the time course of activation of SK

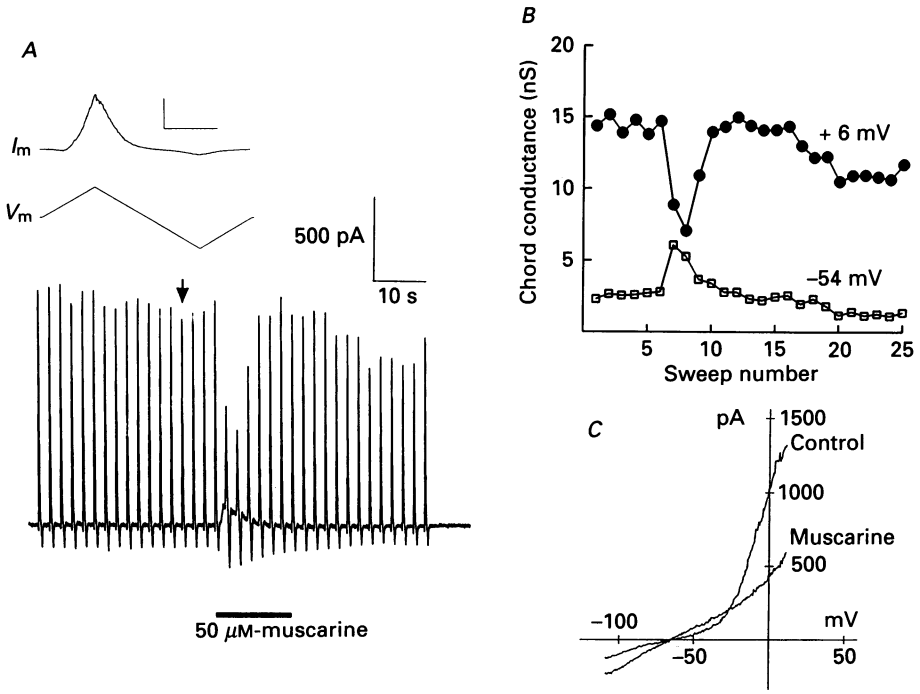

Fig. 10. Muscarine can produce a transient inactivation of voltage-dependent outward current. In *A*, currents were elicited by a voltage ramp waveform (−109 to +11 mV) applied at 0·5 Hz. The two traces on top correspond to a complete 1 s voltage ramp (V_m) and the resulting membrane current (I_m). Vertical calibration bars for these traces are 100 mV (V_m) and 750 pA (I_m). The horizontal calibration bar corresponds to 256 ms. The ramp marked by the arrow (bottom trace in *A*) corresponds to the voltage and current sweeps shown on the top. The lower trace in *A* illustrates the effect of 50 μM-muscarine on a slower time base when applied by U-tube perfusion for the period indicated by the bar. At the holding potential of −49 mV, the typical muscarine-activated transient outward current can be seen. At the peak of the voltage ramp (+11 mV), a large apparent decrease in outward current is seen. Both the conductance increase observed below −40 mV and the apparent conductance decrease at +11 mV have completely waned before the end of the muscarine application. This trace also shows a prolonged decrease in membrane conductance at potentials negative to the holding potential which is observed following muscarine application. Whether this phenomenon represents run-down or a muscarine-initiated effect is not clear. In *B*, the chord conductance calculated at +6 mV and at −54 mV is plotted as a function of sweep number. At the time of maximal inactivation of outward current, the total membrane conductance is almost completely accounted for by the conductance active at −54 mV. There is virtually no voltage-dependent outward current at the peak of the outward current inactivation. Note the prolonged conductance decrease at −54 mV following muscarine application. In *C*, the current–voltage relationship for a ramp before muscarine application and at the time of the maximal muscarine-induced outward current inactivation are shown. Note that in the presence of muscarine there is a net increase in conductance for all potentials below −30 mV and that the membrane conductance is virtually voltage independent from −100 to −20 mV.

current at more negative potentials suggests that the inactivation process occurs, in part, as a consequence of the transient elevation of Ca^{2+}.

With relatively small depolarizing swings during the voltage ramps, the

inactivation of outward current in response to muscarine appeared to wash out during the experiment. By driving the cell to more positive voltages during the voltage ramps, the ability of muscarine to lead to inactivation of outward current was more stable. Furthermore, as seen in Figs 8 and 9, some cells do not exhibit an

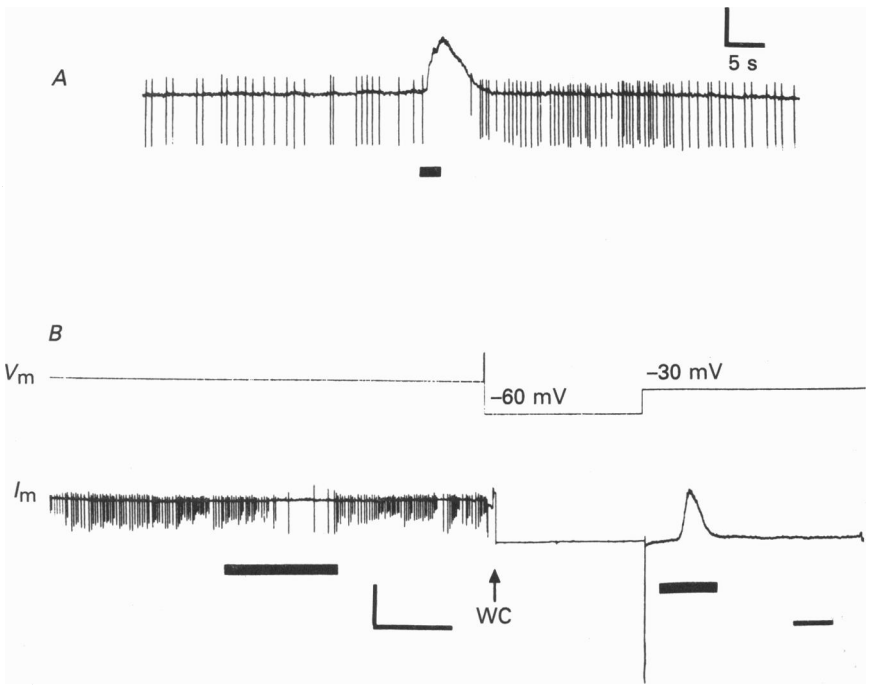

Fig. 11. Muscarine transiently inhibits spontaneous action currents in single rat chromaffin cells. Both *A* and *B* show representative examples of spontaneous action currents recorded with cell-attached pipettes. Bars indicate the period of application of muscarine to each cell. In both *A* and *B*, muscarine inhibits spontaneous action currents and, in *A*, a noticeable outward current associated with muscarine application is observed. In *A*, there is also an increase in spontaneous action potential frequency following the initial suppression of action potentials. In *B*, the effect of muscarine is shown both before and after formation of the whole-cell recording mode (at arrow). Following inhibition of spontaneous action currents in the cell-attached recording, 0·5 μM-musarine elicits a transient outward current when the cell is clamped at −30 mV. V_m, membrane voltage; I_m, membrane current. Vertical calibration: 50 pA; horizontal calibration: in *A*, 5 s; in *B*, 10 s. Note that in *B* there are two different horizontal calibration bars corresponding to times before and after formation of the whole-cell (WC) recording.

obvious inactivation of outward current at the peak of the voltage ramp. Our present results do not provide a full explanation of these observations, but may reflect differences in the amount of cytosolic Ca^{2+} elevation among responses. In cells in which outward currents were activated by depolarizing command steps to +81 mV, run-down of the inactivation phenomenon was not observed. In such cases, muscarine results first in an initial activation of outward current at +81 mV presumably as cytosolic Ca^{2+} is elevated. This is followed by a depression of outward current and then a slow recovery of outward current as the cytosolic level of Ca^{2+}

falls. To exclude the possibility that the apparent inactivation of K^+ current occurs as a secondary consequence of a muscarine-induced inhibition of Ca^{2+} current, the muscarine-induced inactivation was examined in the presence or absence of 5 mM-Co^{2+}. No differences in the ability of muscarine to result in inactivation of outward current were observed (data not shown). Hence, under conditions in which modulation of Ca^{2+} current would be unable to contribute to the elevation of cytosolic Ca^{2+} produced by the depolarizing command steps, the inactivation of outward current is still observed.

In sum, the results indicate the following. Muscarinic AChR stimulation can result in an inactivation of outward current at positive command potentials. The time course of this inactivation mirrors the time course of the Ca^{2+}-dependent activation of SK current at more negative potentials, suggesting that the concentration of cytosolic Ca^{2+} may be involved in the inactivation process. The inactivation process occurs in the absence of Ca^{2+} influx. The inactivation mechanism probably involves a direct effect on the Ca^{2+}- and voltage-dependent K^+ channel.

Effects of muscarine on extracellularly recorded action potentials

The above experiments have documented a rather complex set of cellular phenomena produced by muscarine. Since it seems likely that the relative contribution of particular membrane currents may vary among cells, it is difficult to predict what the expected behaviour of a chromaffin cell might be in response to muscarine. Two kinds of experiments were done to obtain an overview of the kinds of behaviour that unclamped chromaffin cells might exhibit in response to muscarine. Specifically, the effects of muscarine on action currents recorded with a cell-attached patch pipette and on changes in cytosolic Ca^{2+} during prolonged recordings were qualitatively examined.

Previous work indicating that mAChR stimulation results in an increased frequency of spontaneous action potentials in rat chromaffin cells (Brandt *et al.* 1976; Kidokoro *et al.* 1979; Kidokoro & Ritchie, 1980; Akaike *et al.* 1990*a*, *b*) would, at least in part, be unexpected based on the results presented above. To address this issue, cell-attached recordings of spontaneous action currents in single chromaffin cells were investigated. The frequency of action potentials recorded under such conditions is quite variable and there is no single stereotypic type of chromaffin cell behaviour. Many cells are largely silent and fire only if the membrane patch is mildly depolarized or hyperpolarized. In some cases, chromaffin cells appear to exhibit bursts of activity followed by long quiescent periods.

Examples of the effect of muscarine on spontaneous action currents are illustrated in Fig. 11. The primary effect of muscarine is a transient blockade or reduction in action potential frequency (Fig. 11*A* and *B*) which in some cases is correlated with an outward current. In Fig. 11*B*, the inhibition of action potentials is correlated with a subsequent transient activation of outward current observed following formation of the whole-cell recording mode. In some cells, subsequent to the inhibition of action potential firing (Fig. 11*A*), a period of increased action potential frequency is often observed which persists for tens of seconds. These effects are potentially consistent with the voltage-clamp observations as follows. Activation of SK and perhaps some BK current subsequent to a release of cytosolic Ca^{2+} would be

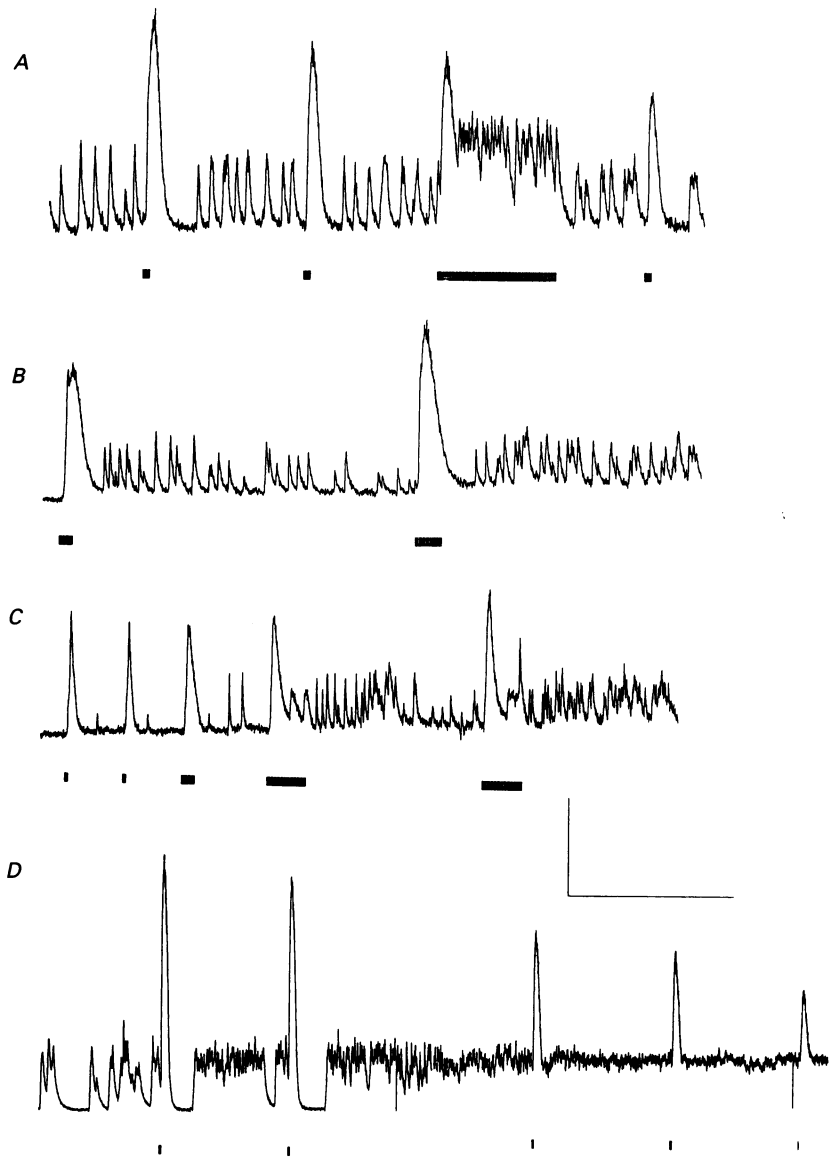

Fig. 12. Effects of muscarine on cytosolic Ca^{2+} in unclamped rat chromaffin cells. In *A*, four applications of 40 μM-muscarine are illustrated. The first, second and fourth applications were for 5 s while the third was for 60 s. Continuous application of muscarine results in a sustained increase in frequency of Ca^{2+} transients. In *B*, two applications of 10 μM-muscarine produce a transient elevation of Ca^{2+} followed by increases in spontaneous Ca^{2+} transient frequency. In *C*, 40 μM-muscarine was applied for two, 5 s, one 20 s and two 60 s applications. An increase in frequency of spontaneous Ca^{2+} transients follows the final two applications. Note also the two secondary transients during the first 60 s muscarine application. In *D*, the response to five 3 s applications of 50 μM-muscarine is shown. Note the switch to a high frequency of spontaneous Ca^{2+} transients which fuse to produce a relatively sustained plateau of elevated Ca^{2+}. Muscarine is still able to trigger additional elevation of Ca^{2+} during this period, although the response runs down. Vertical calibrations: *A*, *B*, *C*: 0·5 F_1/F_2; *D*: 3·0 F_1/F_2; horiontal calibrations: *A*, *B*, 125 s; *C*, *D*: 250 s.

expected to transiently suppress action potential generation. The results provide no single explanation of the increase in action potential frequency. This increase might either reflect removal of inactivation of voltage-dependent currents following a Ca^{2+}-induced hyperpolarization or, alternatively, might reflect small decreases in resting K^+ conductances following muscarine applications, as seen in Fig. 10. We have not examined the effects on membrane currents of muscarine applications longer than about 10 s.

The effects of muscarine on Ca_i^{2+} described earlier focused primarily on cells in which spontaneous changes in Ca_i^{2+} were minimal. However, as seen in Fig. 4, chromaffin cells are capable of exhibiting spontaneous Ca^{2+} transients. Although some cells are primarily quiescent, in other cases cells exhibit complex firing behaviour.

To provide some indication of the complexity of the responses to muscarine in unclamped cells, Fig. 12 illustrates the responses of four different chromaffin cells to muscarine. In Fig. 12*A*, brief application of muscarine produces a transient inhibition of spontaneous Ca^{2+} transients, while during the prolonged application of muscarine an elevated frequency of spontaneous Ca^{2+} transients is seen following the initial transient elevation of Ca^{2+}. In Fig. 12*B*, a cell which is relatively quiescent prior to muscarine application is observed to undergo spontaneous Ca^{2+} transients following the initial transient elevation of Ca^{2+} induced by muscarine. Figure 12*C* illustrates a similar effect. In separate experiments, we have noted that the transient elevation of Ca^{2+} activated by caffeine can also subsequently result in a similar elevated spontaneous Ca^{2+} transient frequency. This would suggest that many of the slowly developing effects of muscarine may only occur as a consequence of Ca^{2+} elevation, rather than as a consequence of mAChR activation. Finally, Fig. 12*D* illustrates the tendency of chromaffin cells to exhibit relatively sporadic spontaneous Ca^{2+} transients and also prolonged bursts of higher frequency Ca^{2+} transients which, because of the slow clearance of Ca^{2+}, appear as a noisy sustained plateau of Ca^{2+} elevation. At present, it is not possible to determine to what extent mAChR activation is necessary for these types of behaviours. However, the results do suggest that, at least in some cases, during prolonged application of muscarine the initial transient elevation of Ca_i^{2+} appears to be followed by an elevated action potential frequency. As argued earlier, the frequency of occurrence of such transients and their sensitivity to 0 Ca^{2+} and 2 mM-Co^{2+} suggest that they largely reflect spontaneous electrical activity of these cells. Such behaviour would be consistent with earlier results describing muscarine-induced increases in action potential frequency (Brandt *et al.* 1976; Kidokoro *et al.* 1979; Akaike *et al.* 1990*a*). Our ability to detect Ca^{2+} transients which may result from spontaneous action potential activity appears at variance with recent work on rat chromaffin cells (Malgaroli, Fesce & Meldolesi, 1990) in which no rapid spontaneous Ca^{2+} transients were observed. The basis for this discrepancy is not clear. Although our results seem most consistent with the idea that spontaneous Ca^{2+} transients result primarily from spontaneous electrical activity, we cannot exclude the possibility that in some cases spontaneous or oscillatory release of Ca^{2+} from intracellular stores may occur (Magloroli *et al.* 1990).

DISCUSSION

Muscarinic receptor activation produces a complex set of conductance changes in rat chromaffin cells. Furthermore, as suggested by the effects of muscarine on spontaneous action currents and cytosolic Ca^{2+} transients, muscarine produces quite complicated effects on patterns of chromaffin cell electrical activity. One effect of muscarine is a transient increase in membrane K^+ conductance. A similar effect has also recently been noted by Kubo & Kidokoro (1989), although the identity of the K^+ current involved was not determined. In addition, nearly coincident in time with the conductance increase, muscarine can produce a transient conductance decrease that is apparent at more positive membrane potentials. We have also noted a more prolonged conductance decrease in some cells which outlasts the application of muscarine, but have not examined this phenomenon here. This latter effect may be similar to a decrease in K^+ current observed in response to muscarine in freshly isolated rat chromaffin cells (Akaike *et al.* 1990*a*, *b*).

Some of the effects of muscarine reported here are consistent with an elevation of Ca_i^{2+} thought to be triggered by IP_3. Muscarine is capable of activating two components of outward current which, based on voltage dependence and pharmacological criteria, appear identical to two components of Ca^{2+}-activated outward current found in these cells (Neely & Lingle, 1992). At potentials negative to about -40 mV, the muscarine-activated current is largely blocked by 200 μM-curare or 200 nM-apamin, and variably sensitive to TEA. Voltage ramps indicate that muscarine-activated current at potentials negative to -40 mV is relatively voltage independent. The results indicate that muscarine-activated current at potentials negative to -40 mV is similar to the Ca^{2+}-activated, voltage-independent K^+ current observed in these cells (Neely & Lingle, 1992). This current appears similar to the so-called SK current observed in a number of other cell types (Romey & Lazdunski, 1984; Blatz & Magleby, 1986; Lang & Ritchie, 1987). Furthermore, this current is also similar to the I_{AHP} current of sympathetic ganglionic neurones (Nohmi & Kuba, 1984; Pennefather *et al.* 1985; Kawai & Watanabe, 1986; Goh & Pennefather, 1987). In addition, at more positive potentials, a voltage-dependent component of muscarine-activated current can be activated. This component is blocked almost completely by 1 mM-TEA and appears identical to the Ca^{2+}- and voltage-dependent K^+ current, often called the BK current, found in these and other cells (Marty, 1981; Barrett *et al.* 1982; Magleby & Pallotta, 1983; Yellen, 1984; Marty & Neher, 1985; Lang & Ritchie, 1987; Ritchie, 1987*a*; Neely & Lingle, 1992).

The present results also show that muscarine produces a transient elevation of cytosolic Ca^{2+} and that both the muscarine-activated currents and the muscarine-induced elevation of Ca_i^{2+} can occur during blockade of Ca^{2+} influx. Thus, in single rat chromaffin cells, mAChR activation appears to result in the release of Ca^{2+} from intracellular pools. Our experiments do not address the nature of the steps linking receptor occupation with release of intracellular Ca^{2+}. Although we have no direct evidence that the currents activated in the presence of muscarine are, in fact, activated by Ca^{2+}, the simplest interpretation of the results is that, as a consequence of an IP_3-mediated release of Ca^{2+} from intracellular stores, two distinct Ca^{2+}-dependent K^+ channels are transiently activated. One expected consequence of the

K^+ channel activation might in some cases be a transient reduction in action potential firing rate. Such an effect is observed.

Time course, inactivation and recovery of muscarine responses

Many qualitative features of the activation of outward current initiated by muscarine in chromaffin cells are quite similar to responses activated by muscarinic receptors in lacrimal glands (Horn & Marty, 1988) and mAChRs expressed in cell lines (Neher, Marty, Fukuda, Kubo & Numa, 1988). The characteristic shift in latency of current (or Ca^{2+} elevation) in response to changes in agonist concentration has been interpreted as indicative of a second messenger-mediated signalling system in which a critical threshold concentration of some intracellular product is necessary in order for the onset of the response to occur (Horn & Marty, 1988). One difficulty encountered in some previous work with responses involving muscarine-induced elevations of IP_3 and Ca_i^{2+} has been the lability of the response to intracellular dialysis (Horn & Marty, 1988). However, with the salines employed here, the ability of muscarine to elicit the release of Ca^{2+} from intracellular stores appears to be well supported, suggesting that the G-protein-mediated activation of phospholipase C (PLC) with the consequent elevation of IP_3 functions normally. Given the dialysis rates for small and large molecules established by Pusch & Neher (1988), the stability over time of the muscarine-activated outward current implies that most diffusible components of the cell would have exchanged at a time when the response is still intact. Although our results cannot exclude the possibility that a critical dialysis-sensitive component is slowly lost during our recordings, the fact that the ability of muscarine to produce elevations of cytosolic Ca^{2+} undergoes some essentially irreversible deterioration even in non-dialysed cells implies that the loss of muscarinic sensitivity may reflect some intrinsic property of the muscarinic response, rather than a limitation of the experimental approach. Such desensitization is not uncharacteristic of muscarinic-mediated processes involving PLC (Llano & Marty, 1987; Maruyama, 1989).

The present results provide little information about the factors that may contribute to the transient nature of the Ca^{2+} elevation and the transient activation of outward current by muscarine. Clearly, the inactivation is not the result of some intrinsic property of the SK current, since SK current exhibits little or no inactivation in the sustained presence of rather high levels of cytosolic Ca^{2+} (Neely & Lingle, 1992). In separate experiments (C. J. Lingle & A. Neely, unpublished), the recovery of the ability of muscarine to elicit either an outward current or an elevation of Ca_i^{2+} can be facilitated by procedures which result in a net influx of Ca^{2+} during the recovery period. This effect suggests that at least some of the transient inactivation of the muscarinic response reflects the slow refilling of intracellular stores with Ca^{2+}.

The transient conductance decrease

Another intriguing aspect of the present results is the pronounced inhibition of Ca^{2+}- and voltage-dependent outward current in response to muscarine. The present results do not directly show that this phenomenon represents the direct inactivation of BK current. However, several observations support this view. During inactivation

of the outward current seen in Fig. 10 virtually all residual membrane conductance is voltage independent and presumably SK current. This indicates that, although cytosolic Ca^{2+} is elevated, there is no BK current. Since the current inactivated by muscarine is apparently voltage dependent and BK current is the major voltage-dependent outward current in rat chromaffin cells, BK current is the best candidate for the current inactivated by muscarine. Furthermore, the reduction of outward current cannot be an indirect effect of Ca^{2+} channel inhibition, since the inactivation of outward current occurs in the absence of Ca^{2+} current. The results do not address the possible mechanism underlying this inactivation of current. However, evidence described in the previous paper (Neely & Lingle, 1992) raised the possibility that the voltage-dependent and TEA-sensitive component of Ca^{2+}-activated current in rat chromaffin cells inactivates during depolarizing commands. The temporal similarity of the activation of SK current and the inactivation of outward current at positive potentials suggests that the elevation of Ca^{2+} may play a critical role in inactivation of outward current during muscarine application. One hypothesis which we are pursuing is that inactivation of BK current in rat chromaffin cells, similar to inactivation of other voltage-dependent currents, is coupled to activation of BK current. As a result, activation of the current either with increased depolarization or by elevation of cytosolic Ca^{2+} will lead to inactivation of the current.

Irrespective of the mechanism of BK inactivation, any process that results in inactivation of BK current would be expected to have profound consequences on action potential duration. Despite the widespread distribution among different cell types of this type of channel (Petersen & Maruyama, 1984), there are only a few reports suggesting that this type of current may be subject to active modulation. In some cases, a cAMP-mediated activation of this current has been reported (Lechleiter, Dartt & Brehm, 1988; Kume, Takai, Tokuno & Tomita, 1989). In contrast, in colonic smooth muscle, a G-protein-mediated suppression of a BK-like current has been described (Cole, Carl & Sanders, 1989; Cole & Sanders, 1989). The physiological consequences of this suppression remain to be determined.

What could be the role of a transient inactivation of the BK current? Of the K^+ currents identified in rat chromaffin cells, BK current is most likely to play an active role in action potential repolarization (Adams, Constanti, Brown & Clark, 1982*c*; Lancaster & Pennefather, 1987; Lang & Ritchie, 1990). Inactivation of this current would be expected to lead to a pronounced prolongation of action potential duration resulting in more Ca^{2+} influx per action potential. However, this effect would seem to be negated by the near-simultaneous activation of SK current which would act as a break to action potential firing. Clear delineation of the roles of BK and SK current in the response to muscarine will require an assessment of the relative extent of activation and inactivation of the two currents and the precise timing of the two phenomena. In addition, any interpretation of the consequences of mAChR activation must also take into consideration the coincident activation of nAChRs which would occur under physiological conditions. Furthermore, the relative contribution of BK and SK current may vary among cells. At present, the results do not provide a clear hypothesis for the functional role of inactivation of K^+ current by muscarine.

Physiological role of activation of outward current

The activation of Ca^{2+}-activated K^+ channels during the initial stages of muscarine action is similar to the responses to secretagogues in a variety of other cell types. In rat clonal pituitary cells (Ozawa & Kimura, 1979; Ozawa, 1981; Dubinsky & Oxford, 1985; Ritchie, 1987*b*; Mollard *et al.* 1988), thyrotrophin-releasing hormone (TRH) initiates an initial activation of Ca^{2+}-dependent K^+ current followed by a secondary increase in action potential frequency. A similar transient activation of outward current occurs in response to muscarine in a number of inexcitable secretory cells (Trautman & Marty, 1984; Horn & Marty, 1988; Gray, 1988; Maruyama, 1989). Despite the widespread occurrence of a hyperpolarizing response during the initial stages of secretagogue action, there is no clear functional role for this type of effect in secretory cells (Petersen & Murayama, 1984). In the case of rat chromaffin cells, the following possibilities can be imagined. First, the resulting hyperpolarization might be critical for removing inactivation of voltage-dependent channels which might then lead to a subsequent period of enhanced cellular excitability. Second, by maintaining the membrane potential at more negative potentials during simultaneous stimulation of nAChRs, the influx of Ca^{2+} through nAChRs or other Ca^{2+}-permeant channels might be enhanced. Third, the efflux of K^+ through Ca^{2+}-dependent channels may be in some way important for some unknown aspects of the exocytotic process. Clearly, it must be considered that mAChR activation would occur synchronously with nAChR stimulation. Any predicted effect of SK current activation on membrane potential must be balanced against the depolarizing effect of nAChR activation.

Effect of muscarine on secretion from rat chromaffin cells

The ability of mAChR activation to contribute to splanchnic nerve-elicited catecholamine secretion remains somewhat unclear in the rat (Wakade, 1981; Wakade & Wakade, 1983), although a clear involvement of mAChR has been demonstrated in some mammalian species (Lee & Trendelenburg, 1967). The differences may, in part, reflect the limitations of methods of measurement of secretion from perfused adrenal glands and the patterns of splanchnic nerve stimulation which have so far been investigated. In contrast, direct mAChR stimulation by ACh or muscarine is clearly quite effective in triggering catecholamine secretion from perfused rat adrenals (Warashina *et al.* 1989). Furthermore, at least a portion of the muscarine-induced secretion persists following removal of extracellular Ca^{2+} (Nakazato *et al.* 1984; Malhotra *et al.* 1988; Misbahuddin & Oka, 1988). Our results support the view that, in the rat, muscarine triggers a robust elevation of cytosolic Ca^{2+} which is independent of Ca^{2+} influx.

The effectiveness of muscarine in elevating submembrane Ca^{2+} in rat chromaffin cells would appear to differ from the situation in bovine chromaffin cells. In the latter system, comparable elevations of total free cytosolic Ca^{2+} produced by either nicotine or muscarine result in relatively weak secretion in the case of muscarine and pronounced secretion in response to nicotine (Cheek, O'Sullivan, Moreton, Berridge & Burgoyne, 1989). Such a result is probably indicative that the muscarine-induced release of Ca^{2+} from intracellular stores in bovine chromaffin cells is not as effective

at elevating submembrane Ca^{2+} as observed in the rat chromaffin cells. Here, the results indicate that the muscarine-induced elevation of cytosolic Ca^{2+}, as assayed by the activation of Ca^{2+}-dependent K^+ current, can reach concentrations which exceed that resulting from direct activation of Ca^{2+} current, even at the voltage eliciting maximal activation of Ca^{2+} current. One assumption involved in the use of Ca^{2+}-activated K^+ current as an assay of submembrane Ca_i^{2+} is that the different types of channels are uniformly distributed on the plasma membrane. At present, there is no information that either supports or negates this assumption. For example, an alternative view might be that SK channels are specifically associated with membrane regions in close proximity to sites of cytosolic Ca^{2+} release, while BK channels are associated with Ca^{2+} channels. Such a situation would render the use of Ca^{2+}-dependent K^+ currents of limited value in comparing the effectiveness of voltage and muscarine in elevating submembrane Ca^{2+}.

On the whole, the present results are consistent with the view that at least one portion of muscarine-induced secretion results from the IP_3-mediated elevation of Ca_i^{2+}. Although the results described here have focused on the transient effects of muscarine application, they also raise the possibility that muscarine-induced modulation of membrane currents may also play a role in producing longer duration changes in the electrical activity of the chromaffin cells. Based solely on studies of secretion from chromaffin cells, there is insufficient information at present to indicate whether muscarine-induced modulation of membrane currents might be of importance to secretion. However, secretion studies typically examine the effects of mAChR stimulation over many minutes. Under such conditions, our results would indicate that any IP_3-mediated Ca^{2+} elevation would contribute to secretion only during the first 30–60 s of agonist application. Thus, if mAChR activation were to contribute to ACh-induced catecholamine secretion at later times, other mechanisms are most probably involved. Recent results on freshly isolated rat chromaffin cells have shown that a decrease in a K^+ current active near chromaffin cell resting potentials can also occur in response to muscarine application (Akaike *et al.* 1990*a*, *b*). Whether this effect would have been observed in the experiments described here is not clear. However, it is possible that such an effect might underlie some of the more prolonged effects of muscarine illustrated in Figs 11 and 12. Together, these results suggest that mAChR activation in rat chromaffin cells produces a number of effects which might influence catecholamine secretion. The extent to which these effects are important in the physiological activation of catecholamine secretion by ACh remains to be determined.

We wish to thank J. Herrington, J. Nerbonne, C. Solaro and J. H. Steinbach for comments on the manuscript. This work was supported by NIH and the Florida Affiliate of the AHA.

REFERENCES

ADAMS, P. R., BROWN, D. A. & CONSTANTI, A. (1982*a*). M-currents and other potassium currents in bullfrog sympathetic neurones. *Journal of Physiology* **330**, 537–572.

ADAMS, P. R., BROWN, D. A. & CONSTANTI, A. (1982*b*). Pharmacological inhibition of the M-current. *Journal of Physiology* **332**, 223–262.

ADAMS, P. R., CONSTANTI, D. A., BROWN, D. A. & CLARK, R. B. (1982*c*). Intracellular Ca^{2+}

activates a fast voltage-sensitive K^+ current in vertebrate sympathetic neurones. *Nature* **296**, 746–749.

AKAIKE, A., MINE, Y., SASA, M. & TAKAORI, S. (1990*a*). Voltage and current clamp studies of muscarinic and nicotinic excitation of the rat adrenal chromaffin cells. *Journal of Pharmacology and Experimental Therapeutics* **255**, 333–339.

AKAIKE, A., MINE, Y., SASA, M. & TAKAORI, S. (1990*b*). A patch clamp study of muscarinic excitation of the rat adrenal chromaffin cells. *Journal of Pharmacology and Experimental Therapeutics* **255**, 340–345.

ALMERS, W. & NEHER, E. (1985). The Ca signal from fura-2 loaded mast cells depends strongly on the method of dye-loading. *FEBS Letters* **192**, 13–18.

BARRETT, J. N., MAGLEBY, K. L. & PALLOTTA, B. S. (1982). Properties of single calcium-activated potassium channels in cultured rat muscle. *Journal of Physiology* **331**, 211–230.

BENHAM, C. D. (1989). Voltage-gated and agonist-mediated rises in intracellular Ca^{2+} in rat clonal pituitary cells (GH_3) held under voltage clamp. *Journal of Physiology* **415**, 143–158.

BLATZ, A. L. & MAGLEBY, K. L. (1986). Single apamin-blocked Ca-activated K^+ channels of small conductance in cultured rat skeletal muscle. *Nature* **323**, 718–720.

BRANDT, B. L., HAGIWARA, S., KIDOKORO, Y. & MIYAZAKI, S. (1976). Action potentials in the rat chromaffin cell and effects of acetylcholine. *Journal of Physiology* **263**, 417–439.

CHEEK, T. R., O'SULLIVAN, A. J., MORETON, R. B., BERRIDGE, M. J. & BURGOYNE, R. D. (1989). Spatial localization of the stimulus-induced rise in cytosolic Ca^{2+} in bovine adrenal chromaffin cells. *FEBS Letters* **247**, 429–434.

COLE, W. C., CARL, A. & SANDERS, K. M. (1989). Muscarinic suppression of Ca^{2+}-dependent K current in colonic smooth muscle. *American Journal of Physiology* **257**, C481–487.

COLE, W. C. & SANDERS, K. M. (1989). G proteins mediate suppression of Ca^{2+}-activated K current by acetylcholine in smooth muscle cells. *American Journal of Physiology* **257**, C596–600.

COLQUHOUN, D., NEHER, E., REUTER, H. & STEVENS, C. F. (1981). Inward current channels activated by intracellular Ca in cultured cardiac cells. *Nature* **294**, 752–754.

DOUGLAS, W. W. (1975). Secretomotor control of adrenal medullary secretion: synaptic, membrane, and ionic requirements in stimulus secretion coupling. In *Handbook of Physiology*, section 7, vol. 6, ed. BLASCHKO, H., pp. 367–385. American Physiological Society, Bethesda, MD, USA.

DOUGLAS, W. W., KANNO, T. & SAMPSON, S. R. (1967). Effects of acetylcholine and other secretagogues and antagonists on the membrane potential of adrenal chromaffin cells: an analysis employing techniques of tissue culture. *Journal of Physiology* **188**, 107–120.

DOUGLAS, W. W. & RUBIN, R. P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *Journal of Physiology* **159**, 40–57.

DUBINSKY, J. M. & OXFORD, G. S. (1985). Dual modulation of K channels by thyrotrophin-releasing hormone in clonal pituitary cells. *Proceedings of the National Academy of Sciences of the USA* **82**, 4282–4284.

DUTAR, P. & NICOLL, R. A. (1988). Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger systems: electrophysiology studies *in vitro*. *Journal of Neuroscience* **8**, 4214–4224.

EBERHARDT, D. A. & HOLZ, R. W. (1987). Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. *Journal of Neurochemistry* **49**, 1634–1643.

FELDBERG, W., MINZ, B. & TSUDZIMURA, H. (1934). The mechanism of the nervous discharge of adrenaline. *Journal of Physiology* **81**, 286–304.

FENWICK, E. M., FAJDIGA, P. B., HOWE, N. B. S. & LIVETTE, B. G. (1978). Functional and morphological characterization of isolated bovine adrenal medullary cells. *Journal of Cell Biology* **76**, 12–30.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982*a*). A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. *Journal of Physiology* **331**, 577–597.

FENWICK, E. M., MARTY, A. & NEHER, E. (1982*b*). Sodium and calcium channels in bovine chromaffin cells. *Journal of Physiology* **331**, 599–635.

GALVAN, M. & SEDLMEIR, C. (1984). Outward currents in voltage-clamped rat sympathetic neurones. *Journal of Physiology* **356**, 115–134.

GOH, J. W. & PENNEFATHER, P. S. (1987). Pharmacological and physiological properties of the after-hyperpolarization current of bullfrog ganglion neurones. *Journal of Physiology* **394**, 315–330.

GRAY, P. T. A. (1988). Oscillations of cytosolic calcium evoked by cholinergic and catecholinergic agonist in rat parotid acinar cells. *Journal of Physiology* **406**, 35–53.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-clamp techniques for high resolution current recording from cell and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HIRANO, T., KIDOKORO, Y. & OHMORI, H. (1987). Acetylcholine dose–response relation and effect of cesium in the rat adrenal chromaffin cell under voltage clamp. *Pflügers Archiv* **408**, 401–407.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *Journal of General Physiology* **92**, 145–159.

KAO, L.-S. & SCHNEIDER, A. S. (1985). Muscarinic receptors on bovine chromaffin cells mediate a rise in cytosolic calcium that is independent of extracellular calcium. *Journal of Biological Chemistry* **260**, 2019–2022.

KAWAI, T. & WATANABE, M. (1986). Blockade of Ca-activated K conductance by apamin in rat sympathetic neurons. *British Journal of Pharmacology* **87**, 225–232.

KIDIKORO, Y., MIYAZAKI, S. & OZAWA, S. (1982). Acetylcholine-induced membrane depolarization and potential fluctuations in the rat adrenal chromaffin cell. *Journal of Physiology* **324**, 203–220.

KIDOKORO, Y. & RITCHIE, A. K. (1980). Chromaffin cell action potentials and their possible role in adrenaline secretion from rat adrenal medulla. *Journal of Physiology* **307**, 199–216.

KIDOKORO, Y., RITCHIE, A. K. & HAGIWARA, S. (1979). Effect of tetrodotoxin on adrenaline secretion in the perfused rat adrenal medulla. *Nature* **278**, 63–65.

KILPATRICK, D. L., LEDBETTER, E. M., CARSON, K. A., KIRSHNER, A. G., SLEPESTIS, R. & KIRSHNER, N. (1980). Stability of bovine adrenal medullary cells in culture. *Journal of Neurochemistry* **35**, 679–692.

KIRPEKAR, S. M., PRAT, J. C. & SCHIAVONE, M. T. (1982). Effect of muscarine on release of catecholamines from the perfused adrenal gland of the cat. *British Journal of Pharmacology* **77**, 455–460.

KUBO, T. & KIDOKORO, Y. (1989). Potassium currents induced by muscarinic receptor activation in the rat adrenal chromaffin cell. *Biomedical Research* **10**, 71–81.

KUME, H., TAKAI, A., TOKUNO, H. & TOMITA, T. (1989). Regulation of Ca^{2+}-dependent K^+-channel activity in tracheal myocytes by phosphorylation. *Nature* **341**, 152–154.

LANCASTER, B. & PENNEFATHER, P. (1987). Potassium currents evoked by brief depolarizations in bull-frog sympathetic ganglion cells. *Journal of Physiology* **387**, 519–548.

LANG, D. G. & RITCHIE, A. K. (1987). Large and small conductance calcium-activated potassium channels in the GH_3 anterior pituitary cell line. *Pflügers Archiv* **410**, 614–622.

LANG, D. G. & RITCHIE, A. K. (1990). Tetraethylammonium blockade of apamin-sensitive and -insensitive Ca^{2+}-activated K^+ channels in the GH_3 pituitary cell line. *Journal of Physiology* **425**, 117–132.

LECHLEITER, J. D., DARTT, D. A. & BREHM, P. (1988). Vasoactive intestinal peptide activates Ca^{2+}-dependent K^+ channels through a cAMP pathway in mouse lacrimal cells. *Neuron* **1**, 227–235.

LEE, F.-L. & TRENDELENBURG, U. (1967). Muscarinic transmission of preganglionic impulses to the adrenal medulla of the cat. *Journal of Pharmacology and Experimental Therapeutics* **158**, 73–79.

LLANO, I. & MARTY, A. (1987). Protein kinase C activators inhibit the inositol trisphosphate-mediated muscarinic current responses in rat lacrimal cells. *Journal of Physiology* **394**, 239–248.

MAGLEBY, K. L. & PALLOTTA, B. S. (1983). Calcium dependence of open and shut interval distributions from calcium-activated potassium channels in cultured rat muscle. *Journal of Physiology* **344**, 585–604.

MALGOROLI, A., FESCE, R. & MELDOLESI, J. (1990). Spontaneous $[Ca^{2+}]_i$ fluctuations in rat chromaffin cells do not require inositol 1,4,5-trisphosphate elevations but are generated by a caffeine- and ryanodine-sensitive intracellular Ca^{2+} store. *Journal of Biological Chemistry* **265**, 3005–3008.

MALHOTRA, R. K., WAKADE, T. D. & WAKADE, A. R. (1988). Vasoactive intestinal polypeptide and muscarine mobilize intracellular Ca^{2+} through breakdown of phosphoinositides to induce

catecholamine secretion. Role of IP_3 in exocytosis. *Journal of Biological Chemistry* **263**, 2123–2126.

MARTY, A. (1981). Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* **291**, 497–500.

MARTY, A. & NEHER, E. (1985). Potassium channels in cultured bovine adrenal chromaffin cells. *Journal of Physiology* **367**, 117–141.

MARTY, A., TAN, Y. P. & TRAUTMANN, A. (1984). Three types of calcium-dependent channel in rat lacrimal glands. *Journal of Physiology* **357**, 293–325.

MARUYAMA, Y. (1989). Activation and desensitization mechanisms of muscarinic current response in single pancreatic acinar cells of rats. *Journal of Physiology* **417**, 343–359.

MISBAHUDDIN, M. & OKA, M. (1988). Muscarinic stimulation of guinea pig adrenal chromaffin cell stimulates catecholamine secretion without significant increase in Ca^{2+} uptake. *Neuroscience Letters* **87**, 266–270.

MOLLARD, P., VACHER, P., DUFY, B., WINIGER, B. P. & SCHLEGEL, W. (1988). Thyrotropin-releasing hormone-induced rise in cytosolic calcium and activation of outward K current monitored simultaneously in individual GH_3B_6 cells. *Journal of Biological Chemistry* **263**, 19570–19576.

NAKAZATO, Y., YAMADA, Y., TOMITA, U. & OHGA, A. (1984). Muscarinic agonists release adrenal catecholamines by mobilizing intracellular Ca^{2+}. *Proceedings of the Japan Academy* B **60**, 314–317.

NEELY, A. & LINGLE, C. J. (1988). Voltage-clamp study of rat chromaffin cells and their response to muscarine. *Society for Neuroscience Abstracts* **14**, 278.

NEELY, A. & LINGLE, C. J. (1990). Muscarine-induced release of intracellular Ca^{2+} results in complex effects on 2 Ca^{2+}-dependent K^+ currents in single rat chromaffin cells. *Biophysical Journal* **57**, 110a.

NEELY, A. & LINGLE, C. J. (1992). Two components of calcium-activated potassium current in rat adrenal chromaffin cells. *Journal of Physiology* **453**, 97–131.

NEHER, E., MARTY, A., FUKUDA, K., KUBO, T. & NUMA, S. (1988). Intracellular calcium release mediated by two muscarinic receptor subtypes. *FEBS Letters* **240**, 88–94.

NOHMI, M. & KUBA. (1984). (+)-Tubocurarine blocks the Ca^{2+}-dependent K^+-channel of the bullfrog sympathetic ganglion cell. *Brain Research* **301**, 146–148.

OZAWA, S. (1981). A biphasic effect of thyrotrophin-releasing hormone on membrane K^+ permeability in rat clonal pituitary cells. *Brain Research* **209**, 240–244.

OZAWA, S. & KIMURA, N. (1979). Membrane potential changes caused by thyrotropin-releasing hormone in the clonal GH_3 pituitary cells and relationships to secretion of pituitary hormone. *Proceedings of the National Academy of Science of the USA* **76**, 6017–6029.

PENNEFATHER, P., LANCASTER, B., ADAMS, P. R. & NICOLL, R. A. (1985). Two distinct Ca-dependent K currents in bullfrog sympathetic ganglion cells. *Proceedings of the National Academy of Sciences of the USA* **82**, 3040–3044.

PETERSEN, O. H. & MARUYAMA, Y. (1984). Calcium-activated potassium channels and their role in secretion. *Nature* **307**, 693–696.

PUSCH, M. & NEHER, E. (1988). Rates of diffusional exchange between small cells and a measuring patch pipette. *Pflügers Archiv* **411**, 204–211.

RITCHIE, A. K. (1987*a*). Two distinct calcium-activated potassium currents in a rat anterior pituitary cell line. *Journal of Physiology* **385**, 591–609.

RITCHIE, A. K. (1987*b*). Thyrotrophin-releasing hormone stimulates a calcium-activated potassium current in a rat anterior pituitary cell line. *Journal of Physiology* **385**, 611–625.

ROLE, L. W. & PERLMAN, R. L. (1980). Purification of adrenal medullary chromaffin cells by density gradient centrifugation. *Journal of Neuroscience Methods* **2**, 253–265.

ROLE, L. W. & PERLMAN, R. L. (1983). Both nicotinic and muscarinic receptors mediate catecholamine secretion by isolated guinea-pig chromaffin cells. *Neuroscience* **10**, 979–985.

ROMEY, G. & LAZDUNSKI, M. (1984). The coexistence in rat muscle cells of two distinct classes of Ca^{2+}-dependent K^+ channels with different pharmacological properties and different physiological functions. *Biochemical and Biophysical Research Communications* **118**, 669–674.

STOEHR, S. J., SMOLEN, J. E., HOLZ, R. W. & AGRANOFF, B. W. (1986). Inositol trisphosphate mobilizes intracellular calcium in permeabilized adrenal chromaffin cells. *Journal of Neurochemistry* **46**, 637–640.

TRAUTMANN, A. & MARTY, A. (1984). Activation of Ca-dependent K channels by carbamylcholine in rat lacrimal glands. *Proceedings of the National Academy of Sciences of the USA* **81**, 611–615.

WAKADE, A. R. (1981). Studies on secretion of catecholamine evoked by acetylcholine or transmural stimulation of the rat adrenal gland. *Journal of Physiology* **313**, 463–480.

WAKADE, A. R. & WAKADE, T. D. (1983). Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and exogenous acetylcholine. *Neuroscience* **10**, 973–978.

WARASHINA, A., FUJIWARA, N. & SHIMOJI, K. (1989). Characteristics of nicotinic and muscarinic secretory responses in the rat adrenal medulla studied by real-time monitoring of catecholamine release. *Biomedical Research* **10**, 157–164.

YELLEN, G. (1982). Single Ca^{++}-activated nonselective cation channels in neuroblastoma. *Nature* **296**, 357–359.

YELLEN, G. (1984). Ionic permeation and blockade in Ca^{2+}-activated K^+ channels of bovine chromaffin cells. *Journal of General Physiology* **84**, 157–186.