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Allelotype of Colorectal Carcinomas

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To examine the extent and variation of allelic loss in a common adult tumor, polymorphic DNA markers were studied from every nonacrocentric autosomal arm in 56 paired colorectal carcinoma and adjacent normal colonic mucosa specimens. This analysis was termed an allelotype, in analogy with a karyotype. Three major conclusions were drawn from this analysis: (i) Allelic deletions were remarkably common; one of the alleles of each polymorphic marker tested was lost in at least some tumors, and some tumors lost more than half of their parental alleles. (ii) In addition to allelic deletions, new DNA fragments not present in normal tissue were identified in five carcinomas; these new fragments contained repeated sequences of the variable number of tandem repeat type. (iii) Patients with more than the median percentage of allelic deletions had a considerably worse prognosis than did the other patients, although the size and stage of the primary tumors were very similar in the two groups. In addition to its implications concerning the genetic events underlying tumorigenesis, tumor allelotype may provide a molecular tool for improved estimation of prognosis in patients with colorectal cancer.

THE INACTIVATION OF TUMOR SUPPRESSOR genes is thought to be important in the development of many human malignancies (*1*). Inactivation of these genes, through deletion or mutation, presumably allows a cell to escape normal growth controls. Only one candidate tumor suppressor gene has been cloned (*2*), but the existence of other such genes has been inferred from experiments in which specific chromosomal regions were found to be deleted in tumors. For example, allelic deletions involving 13q, 11p, and 22q are frequently found in retinoblastomas (*3*), Wilms' tumors (*4*), and acoustic neuromas (*5*), respectively. Although initial experiments concentrated on the loss of individual chromosomal regions from specific tumors, recent reports hint at a greater complexity. In particular, allelic deletions of chromosome 11p or 13q have been reported in different studies of breast cancers (*6*); deletions of 3p, 13q, and 17p have been reported in carcinomas of the lung (*7*); and deletions of 5q, 17p, 18q, or 22q have been reported in colorectal cancers (*8, 9*). To gain a broader perspective on the prevalence of allelic deletions in a common adult tumor, we have studied markers from every nonacrocentric autosomal arm in a large number of human colorectal tumors. We have termed this analysis an allelotype, in analogy with a karyotype.

Two technical developments were necessary for the present study. First, a large number of probes useful for identifying restriction fragment length polymorphisms (RFLPs) have been generated. The variable number of tandem repeat (VNTR) probes are particularly useful for such analyses, because each probe can distinguish the two corresponding parental alleles in a high proportion of normal DNA samples (*10, 11*). Second, colorectal tumors, like many solid tumors, contain variable numbers of nonneoplastic cells that can mask allelic deletions occurring within the neoplastic tumor cell population. A cryostat sectioning method for physically fractionating such tumors to enrich for neoplastic cells has been described (*12*).

DNA was purified from cryostat sections of 56 primary colorectal carcinomas removed at surgery and compared to the DNA from normal colonic tissue of the same patients. Probes detecting RFLPs were used to determine whether one of the two parental alleles detected by each probe was specifically lost in the DNA from the tumor cells. All nonacrocentric autosomal arms were studied; the only genes known to be present on the acrocentric arms (13p, 14p, 15p, 21p, and 22p) are ribosomal. For each of these 39 chromosomal arms, enough probes were used to ensure that the two parental alleles could be distinguished in the normal tissue of at least 20 patients (that is, the informative patients).

Alleles from each chromosomal arm were lost in at least some tumors (Fig. 1A). The frequency of allelic loss varied considerably, however, with alleles from two chromosomal arms (17p and 18q) lost from more than 75% of tumors, alleles from nine arms (1q, 4p, 5q, 6p, 6q, 8p, 9q, 18p, and 22q) lost in 25 to 50% of tumors, and the remaining 28 arms lost in 7 to 24% of the tumors (Fig. 1A). There were 127 examples of allelic deletions in which the patient was informative for markers on both the p and q arms of the chromosome containing the deletion. In 65% of these cases, allelic loss occurred in only one of the two chromosomal arms. The majority of the deletions observed in this study therefore represented subchromosomal events, such as might be mediated by interstitial deletion, mitotic recombination, or gene conversion, rather than loss of a whole chromosome.

The frequency of allelic deletions was also remarkable when viewed from the perspective of individual tumors (Fig. 1B and Table

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1). We defined fractional allelic loss (FAL) in a tumor as the number of chromosomal arms on which allelic loss was observed divided by the number of chromosomal arms for which allelic markers were informative in the patient's normal cells. The median FAL in the 56 tumors studied was 0.20; in other words, alleles were lost from 20% of the evaluable chromosomal arms. In 12 tumors, more than a third of the evaluable chromosomal arms had undergone allelic deletion.

We also determined whether the fraction of allelic loss in individual tumors had any relation to clinical or histologic features. For this analysis, the patients were divided into two groups: those with tumors containing less than the median FAL (group I, FAL <0.2) and those containing greater than the median (group II). Both groups of patients were followed for a period averaging 38 months (Table 2). The sex and age distribution of the two groups of patients were similar, and the average size and extent of invasion (Dukes' classification) of their tumors were nearly identical (Table 2). The prevalence of another genetic alteration (*RAS* gene mutation) that occurs commonly in colorectal tumors (*8, 13, 14*) was identical in the two groups (Table 2). Despite these similarities, the patients with more deletions were significantly more likely to develop recurrent disease (including distant metastases in all but one patient who developed tumor recurrence) than the other group ($P < 0.01$, Table 2). These patients were also significantly more likely to die with or from their cancer ($P < 0.01$, Table 2). There was also a significant relation between allelic deletions and clinical course in the subset of patients with less advanced disease at the time of surgery (Dukes' stage A or B). In 14 such patients with more than the median FAL, 11 (79%) developed recurrent disease (usually distant metastases) postoperatively. Only 2 of 14 stage A or B patients in the low FAL group had tumor recurrence ($P < 0.001$, Fisher's exact test). Thus the measurement of allelic losses might help identify patients with an otherwise relatively favorable prognosis who could benefit from additional therapy.

Table 1. Loss of alleles in individual tumors.

Tumor*	Chromosomal arms on which allelic markers were lost	Number of arms with no loss†	FAL
S7	7q, 18q, 20p	19	0.136
S15	5q, 17p, 18q	17	0.150
S16	17p, 18q	19	0.095
S20	9q, 12q, 17p, 18q, 20q, 22q	17	0.261
S22	1p, 8p, 17p, 18p, 18q	25	0.167
S43	1p, 1q, 3q, 4p, 5q, 11p, 13q, 14q, 17p, 18q	5	0.667
S45	10q, 15q, 17p, 18p, 18q	24	0.172
S50	2p, 2q, 6p, 6q, 8p, 15q, 17p, 17q, 18q, 21q	20	0.333
S51	4p, 14q, 17p, 18q	20	0.166
S59	1q, 4p, 5q, 13q, 17p, 18p, 18q, 19p, 19q	15	0.375
S61	9q, 17p, 18q	17	0.150
S62	17p, 21q	22	0.083
S67	1p, 5p, 5q, 11q, 17p, 18q	23	0.207
S74	4p, 5q, 7q, 11p, 12q, 16p, 16q, 17p, 18q, 19q, 22q	10	0.524
S82	5q	22	0.043
S89	1q	24	0.040
S91	1p, 5q, 10q, 12p, 16q, 17p, 22q	18	0.280
S92		20	0.000
S93	1q, 5q, 6p, 6q, 10p, 15q, 17p, 17q	19	0.296
S96	5q, 9p, 16q, 17p, 22q	21	0.192
S98	2q, 9q, 15q, 17p	21	0.160
S99	13q, 17p, 18q	18	0.143
S103	14q, 17p, 18q	20	0.130
S104		24	0.000
S106	4p, 5q, 8p, 9p, 17p, 17q, 18p, 18q	14	0.364
S108	10q, 17p, 18q, 19q	21	0.160
S109	6p, 6q, 16q, 17p, 19p	17	0.227
S115	5q, 14q, 17p, 17q, 18q, 21q	18	0.250
S119-A	1q, 6p, 6q, 14q, 17p, 18p, 18q, 21q, 22q	14	0.391
S119-D	6q, 9q, 18q	20	0.130
S122	3p, 6p, 6q, 8p, 9p, 9q, 17p, 17q, 18q, 22q	19	0.345
S123	1q, 5q, 6p, 6q, 7p, 7q, 9q, 18q	18	0.308
S124	1q, 2q, 3q, 4q, 6p, 6q, 7q, 9q, 11p, 14q, 17p, 18q, 19q	11	0.542
S126	3q	22	0.043
S133	3p, 5p, 5q, 6p, 6q, 11p, 17p, 17q, 20p	16	0.360
S136	1q, 3p, 16q, 17p, 18q, 19p	23	0.207
S140	4q, 5q, 8p, 12q, 17p, 18q, 19q	18	0.280
S141-A	3q, 7p, 7q, 8p, 10p, 10q, 13q, 14q, 17p, 17q, 18p, 18q, 19p, 19q, 22q	9	0.625
S141-B	8p, 9p, 10p, 11q, 14q, 17p, 18p, 18q, 22q	15	0.375
S153	1q, 7q, 8p, 17p, 18q, 22q	20	0.231
S154	4p, 17p, 18q	21	0.125
S161	1p, 1q, 5q, 8p, 8q, 17p, 18q, 20p, 20q	20	0.310
S162	1q, 5q, 6q, 8p, 8q, 10q, 12p, 17p, 18q, 21q, 22q	14	0.440
S165	5q, 9p, 9q, 13q, 17p	16	0.238
S167	3p, 9q, 14q, 17p, 18p, 18q, 21q, 22q	21	0.276
S168	1q, 5q, 17p, 18q	22	0.154
S170	5q, 18q, 22q	27	0.100
S173		21	0.000
S174	8p, 11p, 11q, 14q, 17p, 18p, 18q	21	0.250
S175	18q, 20p	27	0.069
S177	17p, 18p, 18q	17	0.150
S184	17p, 18q	22	0.083
S190	1p, 3p, 9p, 9q, 12q, 17p, 18p, 18q	19	0.296
S191	2q, 5q, 17p, 19p	21	0.160
PS-6	2p, 11q, 12p, 17p, 18q	17	0.227
PS-12	17p, 18p, 18q	21	0.125

*Two patients (S119 and S141) had two separate tumors. †Number of arms on which DNA from normal tissue demonstrated heterozygosity with one or more allelic markers, but both alleles were retained in tumor DNA.

The allelic losses described in Table 1 are conservative measures of the true extent of allelic loss, for two reasons. First, each probe used was not informative in every case, so that, on average, 24.4 chromosomal arms were evaluable in each tumor out of a total of 39 chromosomal arms studied. Second, subchromosomal deletion events that did not result in loss of the region containing the polymorphic markers would not be detected. The frequency of this latter possibility is difficult to estimate accurately, although studies have shown that large regions of chromosomal arms are involved in many of the allelic deletions that have thus far been observed in tumors, particularly on chromosomes 5, 17, and 18 (*8, 9*).

The variability and frequency of allelic deletions in individual primary tumors noted here is much higher than those noted in previous studies in which RFLPs were analyzed. This difference is likely due to the following reasons: (i) Many more chromosomal positions were examined in the present study than in previous ones; and (ii) the study of DNA isolated from cryostat sec-

tions of tumors allows a more reliable visualization of allelic deletions than the study of DNA isolated from unfractionated tumors. Indeed, our results are not inconsistent with karyotypic and flow cytometric studies of colorectal and other solid tumors, which have often shown a high degree of chromosomal loss or aneuploidy, particularly in more aggressive tumors (*15–18*). RFLP analysis extends the karyotypic analyses in two ways. First, it is often difficult to know whether the changes observed in karyotypes of primary tumors are present in only a portion of the cells, for example, those that are most rapidly dividing. The allelic deletions noted in Fig. 1, however, were all clonal, that is, they were present in at least 80% of the neoplastic cells within the tumor, as assessed by quantitative comparison of the autoradiographs with histologic analysis of the cryostat sections from which the DNA samples were prepared. Hence, the deletions occured in the majority, if not all, of the tumor cells within the carcinomas. Second, it is often difficult in karyotypic studies to ascertain whether chromosomal regions that appear to be lost are actually missing from the cell, as opposed to being masked by translocation to other chromosomes, especially when the karyotype is complex. With RFLP analysis, however, the finding of allelic loss is conclusive proof of the absence of the allele.

An unexpected observation noted during the course of these studies was the occurrence of genetic alterations within the restriction fragments detected by some probes. In five different instances, new bands not observed in the DNA from normal tissue were found in DNA from the corresponding tumor. Each case involved a probe from a different chromosomal arm, and these five probes were all of the VNTR type (Fig. 2, B and C). The size of the new bands in an individual tumor was either decreased (in tumors number S98, S153, and S191) or increased (in tumors S7 and S175) by a similar number of base pairs regardless of the enzyme used (*19*).

Such changes in VNTR sequences have not been observed previously in tumors when probes hybridizing to known chromosomal positions were used. However, there have been reports of changes in "DNA fingerprint" patterns of tumor DNA samples when the samples were analyzed with probes that detect a large number of VNTR sequences simultaneously (*20*). It has been hypothesized that VNTR sequences are "hot spots" for the generation of new alleles (*10, 11*), and our finding that these sequences were altered in five different cases supports this hypothesis. Although the occurrence of changes in DNA fragments containing VNTR sequences was rare (five alleles altered out of 2900 VNTR alleles examined), no rearrangements of fragments without VNTR sequences were observed in our study (3100 alleles were examined with non-VNTR probes).

What is the significance of the multiple genetic alterations described in this study? Two explanations are possible for the physiologic basis of a clonal genetic alteration such as an allelic deletion or change in VNTR. First, it is possible that the genetic alteration in-

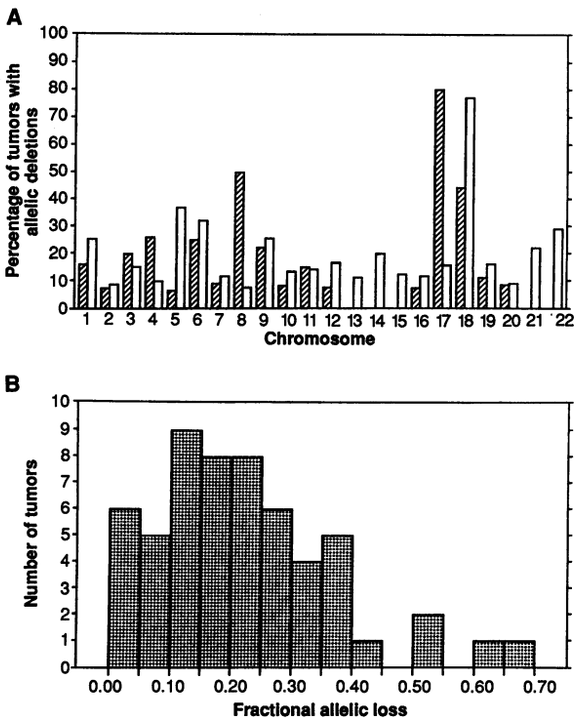

Fig. 1. (A) Frequency of allelic deletions in individual chromosomal arms. Allelic deletions were evaluated with RFLP analyses, examples of which are in Fig. 2. DNA from paired normal colonic mucosa and tumor tissues was cleaved with one of three enzymes (Taq I, Msp I, or Hind III), and evaluated with probes from each nonacrocentric autosomal arm. The probes used are listed (*23*), together with references describing their derivation and polymorphism patterns. Only informative tumors, that is, those in which DNA from the normal tissue exhibited a heterozygous pattern for one or more allelic markers from the indicated chromosomal arm, were used to determine allelic loss frequencies. The number of tumors informative for each chromosomal arm is listed in (*23*). An allelic loss was scored if an RFLP fragment present in normal DNA was lost in at least 80% of the neoplastic cells, as assessed by comparison of the autoradiographs with histologic evaluation of the cryostat sections from which the tumor DNA was purified. Open bars, q arm; hatched bars, p arm. (**B**) Frequency of allelic deletions in individual tumors. The FAL in each tumor was defined as the number of chromosomal arms on which allelic loss was observed divided by the number of chromosomal arms for which allelic markers were informative. The chromosomal arms on which the allelic deletions occurred in each tumor are listed in Table 1.

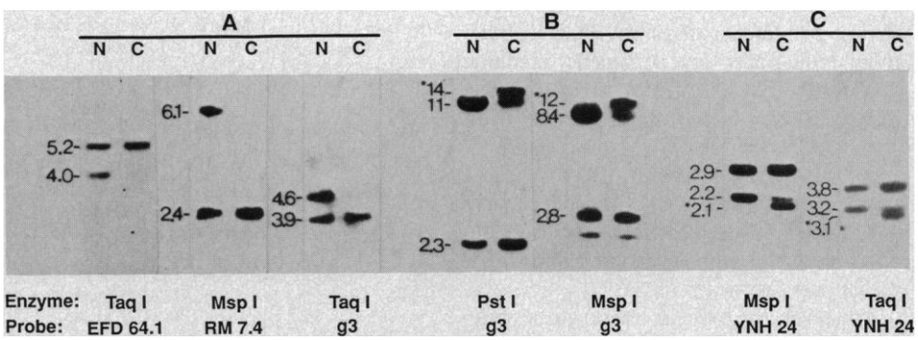

Fig. 2. (**A**) Examples of allelic deletions. DNA from normal (N) and carcinoma (C) tissues of patient S141 (tumor A) was cleaved with restriction endonucleases, and the fragments were separated by electrophoresis and transferred to nylon filters. The filters were incubated with the indicated radioactive probes. Sizes (in kilobases) of the polymorphic restriction fragments are shown on the left of each autoradiograph. With probes RM 7.4 and g3, the larger allele was lost from the tumor; with probe EFD 64.1, the smaller allele was lost. (**B** and **C**) New fragments detected in colorectal tumors with VNTR probes. Autoradiographs of DNA blots prepared as described in (A) are shown. For each N-C pair, the results of digestion with two different enzymes are shown, and the probe is indicated. (B) Patient S7 and (C) patient S191. Sizes (in kilobases) of the major polymorphic restriction fragments are shown on the left of each autoradiograph, and the new fragments in the tumor samples are marked with asterisks. Areas of tumors containing a high proportion of neoplastic cells were isolated as described, and 12-μm-thick-cryostat sections of these areas were used to prepare DNA (*12*). Grossly normal colonic mucosa adjacent to the tumors was obtained from each patient and used to prepare control DNA. DNA purification, restriction endonuclease digestion, electrophoresis, DNA transfer, and DNA hybridization were performed as described (*12, 24*).

Table 2. Relation of FAL to clinical and histologic features. In comparing the means for group I with those of group II, age, follow-up period, tumor size, and Dukes' classification were nonsignificant on the basis of the *t* test. For the comparisons of percentages, *RAS* was nonsignificant and tumor recurrence and death were significant on the basis of the Fisher exact test ($P < 0.01$).

Group*	FAL (mean)	Number of patients†	Age (years)	Follow-up period‡ (months)	Tumor size (cm)	Dukes' class.§	*RAS* mutation‖ (%)	Tumor recurrence¶ (%)	Death# (%)
I	0.11	27	67	38	5.3	2.3	52	30	26
II	0.32	25	67	38	5.6	2.4	52	68	64

*Group I patients had tumors with an FAL less than the median value (0.2) of the 56 tumors listed in Table 1; group II patients had tumors with an FAL greater than 0.2. †All patients from Table 1 with a single carcinoma were included. ‡Mean follow-up period in patients who survive is listed. The mean follow-up period in all patients combined (that is, those who are still alive plus those who died) was 31 and 17.5 months for group I and II patients, respectively. §Dukes' classification scored as 1.0 for Dukes' A tumors (confined to muscularis propria), 2.0 for Dukes' B tumors (extension through muscularis propria), and 3.0 for Dukes' C tumors (metastatic to regional lymph nodes). ‖*RAS* gene mutations in this group of tumors were reported in (*8*) and (*13*). ¶Distant metastases developed in all except one patient who developed tumor recurrence. #Death with or from carcinoma. An additional 6 and 12% of group I and II patients, respectively, died without definite evidence of recurrent carcinoma.

cludes or is linked to a gene whose product can affect neoplastic growth. According to this explanation, there are numerous suppressor genes present throughout the genome, and the inactivation of any of them through deletion could have incremental effects on the regulation of cell growth. Such genes may not require inactivation of both parental alleles to promote a neoplastic effect, but, as suggested (*8, 21*), such effects may be exerted when the genes are present at only half the normal copy number. The behavior of solid tumors may in large part depend on the balance of such genes, and the heterogeneity in allelic deletions noted here may underlie the well-documented heterogeneity of tumors with regard to invasiveness, responses to therapy, and other biologic properties (*22*). Alternatively, at least some of the clonal alterations in colorectal tumors may have no intrinsic effect on cell growth but may coincidentally occur during the same mitosis in which another more biologically potent genetic alteration also occurs. For example, it is possible that many alleles are deleted simultaneously in an abnormal mitosis in which many chromosomes segregate aberrantly. Because allelic losses are irreversible, these "passenger" losses will remain as the cell proliferates and eventually becomes the predominant clone in the tumor population.

Whatever the role of these allelic deletions, their multiplicity must be taken into account in any model for the genetic origin of human tumorigenesis. Moreover, they prompt significant caution in interpreting the significance of individual allelic deletions or other genetic alterations occurring in these and other tumors. The results shown in Fig. 1 and Table 1 emphasize that such individual changes represent, at most, single components in a complex tumor evolutionary process. On the other hand, the data in Table 2 suggest that recognition and measurement of the accumulated genetic changes, such as provided by the allelotype, may lead to useful molecular correlates of tumor behavior.

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19. In each case, at least six enzymes were used, and the same change was seen whenever the fragments could be resolved (with some enzymes and probes, the fragments present in normal DNA were too large to be resolved). Examples for two tumors are given in Fig. 2, B and C. In addition, new fragments were seen with probe YNA 13 in patient S153, with probe EKDMA2 in patient S95, and probe CMM6 in patient S175.
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Regulation of Calcium Concentration in Voltage-Clamped Smooth Muscle Cells

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The regulation of intracellular calcium concentration in single smooth muscle cells was investigated by simultaneously monitoring electrical events at the surface membrane and calcium concentration in the cytosol. Cytosolic calcium concentration rose rapidly during an action potential or during a voltage-clamp pulse that elicited calcium current; a train of voltage-clamp pulses caused further increases in the calcium concentration up to a limit of approximately 1 μ*M*. The decline of the calcium concentration back to resting levels occurred at rates that varied with the calcium concentration in an apparently saturable manner. Moreover, the rate of decline at any given calcium concentration was enhanced after a higher, more prolonged increase of calcium. The process responsible for this enhancement persisted for many seconds after the calcium concentration returned to resting levels. Thus, the magnitude and duration of a calcium transient appear to regulate the subsequent calcium removal.

IN SMOOTH MUSCLE, AS IN SKELETAL and cardiac muscle, cytosolic calcium concentration ($[Ca^{2+}]$) is the principal regulator of contraction (*1*). In contrast to the two types of striated muscle (*2, 3*), however, the relation between membrane electrical events and the rise and fall of cytosolic $[Ca^{2+}]$ has not been examined directly in smooth muscle on the time scale of physiological events. We have measured $[Ca^{2+}]$ with high-time resolution in voltage-clamped single smooth muscle cells, thus allowing simultaneous monitoring of Ca^{2+} currents (I_{Ca}) and changes in cytosolic $[Ca^{2+}]$. Furthermore, because Ca^{2+} influx could be rapidly initiated and terminated by using the voltage-clamp technique, we could study the processes responsible for restoring cytosolic $[Ca^{2+}]$ to resting levels. These techniques have enabled us to describe several kinetic and regulatory characteristics of the processes that control the $[Ca^{2+}]$, and thus contraction, of smooth muscle.

We used single smooth muscle cells isolated from toad stomach (*4*) that were loaded with the fluorescent Ca^{2+} indicator fura-2 (*5, 6*). Cytoplasmic $[Ca^{2+}]$ was measured with a high-time resolution microfluorimeter (*7*). Membrane potential and ionic currents were monitored with a single microelectrode (*8*) rather than a patch electrode to avoid dialysis of the cytoplasm and thus minimize the loss of factors that might be important in buffering or regulating the $[Ca^{2+}]$. In 20 m*M* extracellular Ca^{2+}, the resting cytosolic $[Ca^{2+}]$ in relaxed, microelectrode-impaled smooth muscle cells averaged 226 ± 22 n*M* (mean ± SEM; n = 17), a value similar to that reported in unimpaled cells (*9*).

Transient elevations in the cytosolic $[Ca^{2+}]$ occurred in response to action potentials (APs) induced at the offset of a hyperpolarizing current or in response to depolarizing command pulses under voltage clamp (Fig. 1). The average increase in $[Ca^{2+}]$ in response to either type of stimulation was 379 ± 32 n*M* (n = 25). During an AP, in which inward current is carried exclusively by Ca^{2+} (*10*), the rise in $[Ca^{2+}]$ occurred most rapidly starting at a potential of about −20 mV, and this rapid phase ended after the AP reached its peak (Fig. 1, A and B). Typically, a slow, small rise occurs as the potential rises through the range of −35 to −20 mV. During a depolarizing voltage-clamp command pulse, the rise in $[Ca^{2+}]$ was roughly proportional to the cumulative inward I_{Ca} (Fig. 1D) for the initial 100 to 200 ms (*11*), a period when the magnitude of the inward I_{Ca} was large. This observation, coupled with the fact that the $[Ca^{2+}]$ continues to rise throughout the AP suggests that, unlike skeletal muscle, the rise in cytosolic $[Ca^{2+}]$ depends strongly on Ca^{2+} influx through voltage-dependent sarcolemmal Ca^{2+} channels. Toward the end of a 2-s command pulse, the I_{Ca} declined, and the $[Ca^{2+}]$ either rose slowly or started to fall. However, in all cases the $[Ca^{2+}]$ declined more rapidly upon repolarization, suggesting that Ca^{2+} influx persisted as long as the cell was depolarized.

Although the rise in $[Ca^{2+}]$ appeared roughly proportional to the integrated inward I_{Ca}, the amount of Ca^{2+} entry determined by integrating the I_{Ca} far exceeded that calculated from the measured free $[Ca^{2+}]$ change. For example, integration of the I_{Ca} during the first 50 ms of the command pulse in Fig. 1D indicated a Ca^{2+} entry sufficient to elevate the $[Ca^{2+}]$ by 19 μ*M*, 40-fold greater than the measured 0.47 μ*M* change in the free $[Ca^{2+}]$ (*12*). Thus, most of the Ca^{2+} that crosses the sarcolemma must be binding to Ca^{2+} buffers, a conclusion consistent with current estimates of the physiological Ca^{2+} buffering capacity in smooth muscle (*13*).

A train of depolarizing command pulses produced summation of the $[Ca^{2+}]$ (Fig. 2). However, each successive pulse resulted in a smaller increase in $[Ca^{2+}]$, and a steady-state ceiling was reached. This ceiling averaged 856 ± 57 n*M* (n = 20), similar to the value obtained in response to cholinergic agonists or high K^+ concentrations in non–voltage-clamped cells (*14*). Several factors appear to be responsible for this ceiling. Peak I_{Ca} decreased with successive voltage-clamp pulses (Fig. 2B), presumably because of the inactivation of Ca^{2+} channels (*15*). Furthermore, as discussed below, the processes responsible for lowering the $[Ca^{2+}]$ are accelerated after a higher intracellular $[Ca^{2+}]$ is achieved.

On termination of a depolarizing command pulse, the I_{Ca} was also terminated, and the processes responsible for Ca^{2+} removal from the cytosol could be studied in isolation. The relation between the rate of

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